University of Alberta

The Molecular Basis of Human Cholesterol-7α-hydroxylase Regulation by Thyroid Hormone

by

Victor A.B. Drover ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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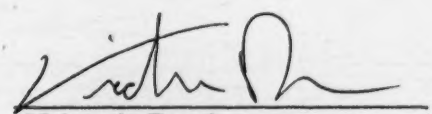

22 Lear's Road
Corner Brook, Newfoundland
Canada A2H 1W3

June 14, 2001
Date Submitted

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "The Molecular Basis of Human Cholesterol-7α-hydroxylase Regulation by Thyroid Hormone" submitted by Victor A. B. Drover in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dr. Luis Agellon

Dr. Jason Dyck

Dr. Mark Glover

Dr. Ruth McPherson

Dr. Dennis Vance

June 14, 2002
Date Thesis Approved

DEDICATION

.....for my parents, Bruce and Linda

ABSTRACT

Bile acids are amphipathic detergents synthesized from cholesterol in the liver. The majority of bile acid biosynthesis is determined by the activity of the liver-specific, microsomal enzyme cholesterol 7α-hydroxylase (cyp7a). Cyp7a activity is regulated primarily *via* induction or repression of cholesterol 7α-hydroxylase gene (*CYP7A1*) expression. Interestingly, some aspects of cyp7a regulation are species-specific. For instance, thyroid hormone (T_3) administration in rodents stimulates cyp7a activity. In contrast, cyp7a activity and bile acid synthesis can be repressed in hyperthyroid humans. In addition, the activity of the human *CYP7A1* gene promoter is reduced by T_3 in human hepatoma cells in the presence of the thyroid hormone receptor (TR). Thus, the goal of this thesis is to determine the molecular basis of T_3-dependent repression of the human *CYP7A1* gene promoter *in vitro* and to examine the ability of T_3 to regulate the gene *in vivo*.

Using DNAse I footprinting and electrophoretic mobility shift assays, two promoter sequences bound by TR in the human *CYP7A1* gene were identified. Functional analyses of human cyp7a1 promoter-reporter gene chimeras containing mutations of these TR binding sites revealed that only one of these two sequences was required for T_3-dependent repression of promoter activity. Preliminary structure-function experiments using a mutant TR suggest that transcriptional coregulators are important for the ability of T_3 to repress the activity of the human *CYP7A1* gene promoter. In contrast, Site III in the murine promoter was not bound by TR and activity of the murine promoter was not affected by T_3.

To test the effect of T_3 on the regulation of human cyp7a *in vivo*, transgenic mice were created with a human genomic DNA fragment containing the *CYP7A1* gene. T_3 was able to repress human *CYP7A1* gene expression in male transgenic mice consistent with the *in vitro* results. However, cyp7a enzyme activity was paradoxically increased by T_3 in these mice. The ability of the murine liver to increase cyp7a enzyme activity despite reduced cyp7a mRNA abundance suggests that increased bile acid synthesis is a necessity in mice during hyperthyroidism.

In conclusion, these results demonstrate that T_3 can repress human *CYP7A1* gene expression *in vivo* and that the molecular basis of this effect is likely the binding of TR to Site III in the *CYP7A1* gene promoter.

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Daisy ... were it not for you, I may not have had the opportunity to write these words. Your support and patience are the pillars upon which I have stood. Without you, this work is a transparent husk without purpose or direction. Thank-you, my Rani.

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List of Abbreviations

ABCA1	ATP binding cassette transporter protein A1
apoA-1	apolipoprotein A-I
apoB	apolipoprotein B
BAC	bacterial artificial chromosome
CA	cholic acid
CAT	chloramphenicol acetyltransferase
CDCA	chenodeoxycholic acid
cyp27	cholesterol 27-hydroxylase
cyp39	24-hydroxycholesterol 7α-hydroxylase
cyp7a	cholesterol 7α-hydroxylase
Cyp7a1	gene encoding cholesterol 7α-hydroxylase
cyp7b	oxysterol 7α-hydroxylase
cyp8b1	sterol 12α-hydroxylase
dex	dexamethasone
DBP	albumin site D-binding protein
DEPC	diethyl pyrocarbonate
DR+1	direct repeat separated by 1 nucleotide
DR+4	direct repeat separated by 4 nucleotides
EHC	enterohepatic circulation
EMSA(s)	electrophoretic mobility shift assay(s)
F_0	transgenic founder mouse
FXR	farnesoid X receptor
FXRE	farnesoid X receptor response element
GCA	glycocholic acid
GCDCA	glycochenodeoxycholic acid
GR	glucocorticoid receptor
HDL	high density lipoprotein
HDL-C	HDL-associated cholesterol
HMGR	HMG-CoA reductase
HNF-4	hepatocyte nuclear factor 4

IR+1	inverted repeat separated by 1 nucleotide
kb	kilobasepair
LDL	low density lipoprotein
LDL-C	LDL-associated cholesterol
LDLR	LDL receptor
LRH-1	liver receptor homolog 1
LRHRE	liver receptor homolog 1 response element
LXR	liver X receptor
non-tg	non-transgenic
nt	nucleotide
PCR	polymerase chain reaction
PPAR	peroxisome proliferator activated receptor
RH7777	McArdle RH7777 hepatoma cells
RT-PCR	reverse transcriptase-PCR
RXR	retinoid X receptor
SHP	small heterodimeric partner
T_3	3',3,5,-triiodothyronine
T_4	3',5',3,5,-tetraiodothyronine
TCA	taurocholic acid
Tg	transgenic
TG	thyroglobulin
TMCA	tauro-β-muricholic acid
TPC	total plasma cholesterol
TR(s)	thyroid hormone receptor(s)
TRE	idealized thyroid hormone response element
VLDL	very low density lipoprotein
VLDL-C	VLDL-associated cholesterol

Note on nomenclature: In general, gene symbols will be written in *italicized* typeface and proteins will not be italicized. For instance, the cholesterol 7α-hydroxylase enzyme will be denoted cyp7a. Different symbols will be used to denote the genes encoding cyp7a in different species: *CYP7A1* (uppercase, italicized) will be used to denote the human gene, *Cyp7a1* (sentence case, italicized) will be used to denote non-human genes. When referring to the gene without reference to a specific species or when referring to a promoter-reporter gene chimera, cyp7a1 (lowercase, non-italicized) will be used.

Chapter 1

INTRODUCTION

1.1 *Bile*

Following a meal, dietary components move through the digestive system and are catabolized to smaller components by gastric and pancreatic enzymes. For instance, lipids such as triacylglycerol are acted upon by pancreatic lipase to produce 2-mono-acylglycerol and free fatty acids. These smaller components, when dissolved, can then be absorbed by the small intestine. However, many lipids are not soluble in the aqueous environment of the small intestine. To solublize lipids, the body secretes bile into the intestinal lumen where it aids to emulsify dietary lipids, thus facilitating their absorption by the intestine.

Bile is a complex mixture consisting primarily of bile salts, cholesterol, and phospholipids[1] and is formed by the secretion of cholesterol and bile salts into the canalicular spaces between hepatocytes (Figure 1-1)[2]. A network of tube-like structures lined by specialized cells called cholangiocytes 'funnel' away from the canaliculi to form the biliary tree and bile ducts. Bile salts extract phospholipids from the canalicular and cholangiocyte membranes and form mixed micelles with cholesterol, thereby completing bile formation[3].

1.2 *Enterohepatic Circulation of Bile Acids*

Bile salts, commonly called bile acids, are synthesized exclusively in the liver and are transported to the intestine *via* the bile ducts. During the emulsification and uptake of fats, bile acids are efficiently recovered by the intestine and return to the liver *via* the portal blood to be reused for bile

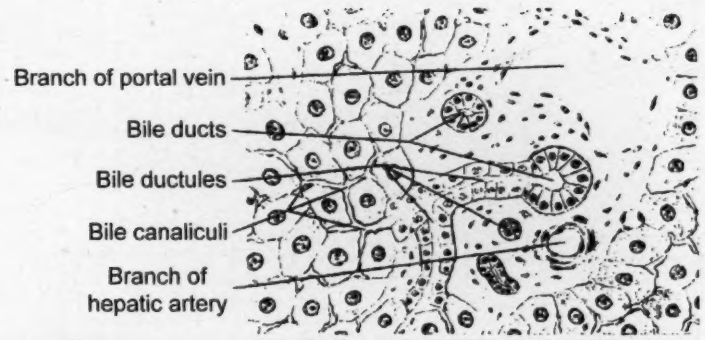

Figure 1-1. HISTOLOGICAL SECTION OF THE LIVER. *Taken from Netter (1995)*[2].

formation. This cycling of bile acids between the liver and the intestine is known as the enterohepatic circulation (EHC) and permits each bile acid molecule to be used several times for each meal[1,3]. This process requires the concerted efforts of a number of proteins to transport the bile acids between intra- and intercellular spaces. The transporters most commonly associated with bile acid transport across cellular membranes are shown in Figure 1-2. The bile salt export pump secretes bile acids into the canalicular spaces. These bile acids are recovered by the enterocyte through the actions of the apical sodium bile acid transporter and are secreted into the portal blood by an unknown protein. Finally, bile acids return to the liver via the sodium taurocholate cotransporting polypeptide.

1.3 Structure and Function of Bile Acids

Bile acids are derived from cholesterol but differ from this substrate in 4 important ways: i) hydroxyl groups are added at position 7 and/or position 12, ii) the 5-6 double bond is saturated, iii) the 3β-hydroxyl group is epimerized to a 3α-

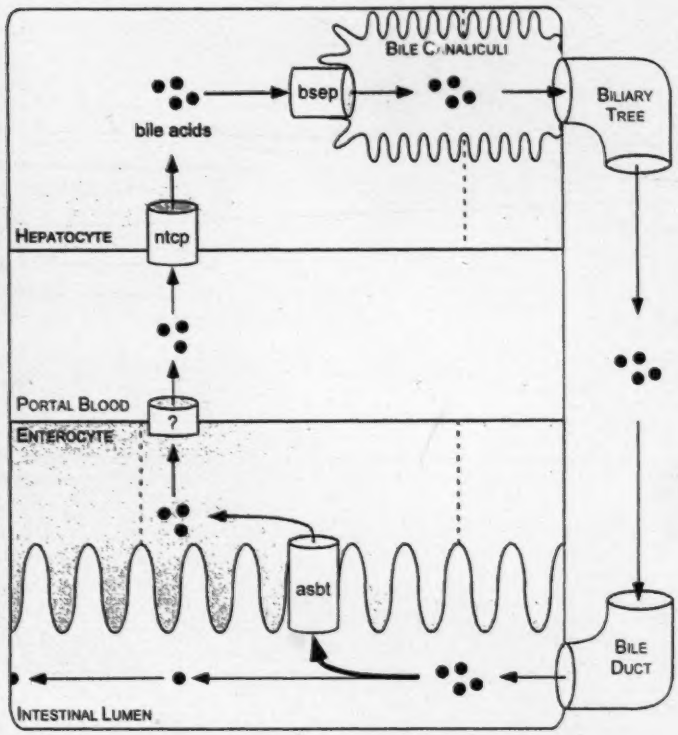

Figure 1-2. THE ENTEROHEPATIC CIRCULATION OF BILE ACIDS. Bile acids are secreted into the canalicular space between hepatocytes by the bile salt export pump (bsep). Bile acids reach the intestine via the bile ducts and most are recovered by the enterocytes through the apical sodium bile acid transporter (asbt). Bile acids are pumped into the portal blood (by an as yet unidentified transporter, indicated by '?') and recovered by hepatocytes by the sodium taurocholate cotransporting polypeptide (ntcp). Arrows indicate the direction of bile acid transport within the enterohepatic circulation and/or loss from the body.

hydroxyl and (iv) the side chain is truncated by 3 carbons and is conjugated to an amino acid (Figure 1-3). These *de novo* synthesized bile acids are termed primary bile acids and fall into two categories, cholic acids (CA, a 3α, 7α, 12α-trihydroxy bile acids) or chenodeoxycholic acids (CDCA, a 3α, 7α-dihydroxy bile acids).

After secretion into the intestinal lumen as bile, the amino acyl moiety of most primary bile acids is deconjugated. These 'free' bile acids can be converted to secondary bile acids *via* a 7-dehydroxylation activity produced by intestinal bacteria[3]. Both primary and secondary bile acids are absorbed by the intestine and cycle together through the EHC. However, free bile acids are reconjugated in the liver prior to secretion into bile. In humans, the most abundant bile acids are

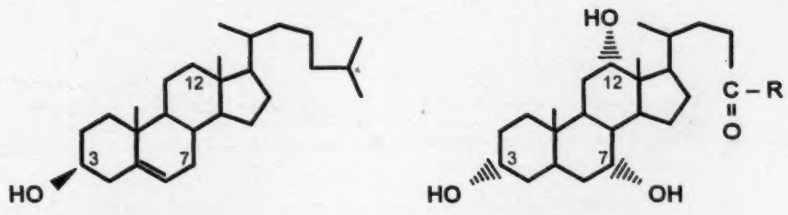

Figure 1-3. THE STRUCTURES OF CHOLESTEROL AND BILE ACIDS. Chemical structures of cholesterol and a generic bile acid are shown. The orientation of the hydroxyl groups is indicated. 'R' represents the conjugated amino acid of the bile acid.

glycocholic (GCA) acid and glycochenodeoxycholic acid (GCDCA) with smaller amounts of their 7-dehydroxylated metabolites (Table 1-1). In rodents, taurocholic acid (TCA) is the predominant bile acid with tauro-β-muricholic acid (TMCA; derived from chenodeoxycholic acid) being the next most-abundant.

Bile acids are the most effective detergents in bile and have the greatest impact on the ability of bile to solublize dietary lipids. This detergent property is a result of the amphipathic nature of bile acids. As shown in Figure 1-3, the four-ringed sterol backbone imparts hydrophobic character to the bile acid. In contrast, α-hydroxylation and conjugation provide hydrophilic character. In

Table 1-1. CHARACTERISTICS OF ABUNDANT BILE ACIDS IN HUMANS AND RODENTS.

Name	Hydroxyl Groups	Amino Acid
HUMAN		
Glycocholic Acid (GCA)	3α, 7α, 12α	Glycine
Glycochenodeoxycholic Acid (GCDCA)	3α, 7α	Glycine
RODENT		
Taurocholic Acid (TCA)	3α, 7α, 12α	Taurine
Tauro-β-muricholic Acid (TMCA)	3α, 6β, 7β	Taurine

Each systematic name is followed by its common abbreviation in parentheses. The position and orientation of each hydroxyl groups are shown, as well as the conjugated amino acid moiety.

addition, the polar groups of bile acids are synthesized such that they all face the same plane of the molecule. Thus, bile acids are perfectly suited to act as detergents. The current model of bile acid action[1,3] describes rod-shaped micelles of fatty acids and/or phospholipids with bile acids embedded into the micelle surface. In this configuration, the hydrophobic protons of the bile acids can interact with the non-polar lipids (i.e the hydrocarbon chains of fatty acids and phospholipids). The hydrophilic groups of the bile acids are directed away from the micelle and interact with the charged lipid head groups and the aqueous environment, thereby emulsifying the lipid. These mixed micelles can also accommodate amphipathic cholesterol molecules.

As noted above, bile acids are conjugated to either taurine or glycine prior to secretion into bile. Amino acid conjugation increases bile acid solubility at acidic pH and reduces precipitation by calcium[4]. These properties are important as the duodenum can be quite acidic[5] while calcium concentrations can be high in the gallbladder[6] where bile is stored and concentrated in many vertebrates. In addition, conjugation makes bile acids impermeable to cell membranes as the amino acyl moiety is fully ionized at physiological pH. This allows for bile acid concentrations in the intestinal lumen to be maintained above the critical micelle concentration which is essential for lipid emulsification. Finally, membrane impermeability contributes to bile flow in the biliary tree by creating an osmotic force and drawing water from the surrounding cells. Thus, conjugation is required for many bile acid functions.

Bile acids have a number of other well characterized functions in addition to emulsification of dietary lipids. Bile acid synthesis is irreversible and thus represents a catabolic endpoint for cholesterol. Bile acids also contribute to cholesterol excretion *via* the stimulation of biliary cholesterol secretion and the solubilization of cholesterol in the biliary tract. Phospholipid secretion into bile is achieved by similar mechanisms. Further, bile acids are natural ligands for a transcription factor and can thus regulate gene expression (see Section 1.4.3(i) and Figure 1-6). However, there are additional properties of bile acids that are not well characterized and require further investigation[3]. For instance, bile acids may be involved in the stimulation of intestinal motility as well as preventing adhesion of intestinal flora to the absorptive surface of the enterocyte (the cells of the intestine). Bile acids can also solublize polyvalent metals such as iron and may aid in their absorption by the intestine. Thus, bile acids serve many physiologic roles in addition to intestinal lipid absorption.

1.4 Bile Acid Biosynthesis

Like many lipids, cholesterol displays poor aqueous solubility and cannot by readily excreted in urine. Through the concerted efforts of at least 11 intracellular enzymes, the bile acid biosynthetic pathway converts cholesterol into an aqueous-soluble molecule. As such, bile acid biosynthesis is the major catabolic route for cholesterol and the manipulation of this process has long been investigated as a means to reduce plasma cholesterol. The following sections describe the chemical conversion of cholesterol into bile acids, as well as the

known regulatory mechanisms which govern the relative rates of bile acid production.

1.4.1 Intermediates in bile acid synthesis – Bile acid synthesis from cholesterol requires a series of chemical conversions including hydroxylation, saturation, epimerization and truncation (Figure 1-3). Each of these chemical conversions requires multiple enzymes and at least ten intermediates are formed in the process. In addition, the enzymes exist in four subcellular locations (see Section 1.4.2) thereby necessitating the intracellular transport of these intermediates. The summary of bile acid synthesis shown in Figure 1-4 highlights the intermediates which move between these subcellular locations to reach the subsequent enzymes in the biosynthetic pathway [reviewed in Russell and Setchell, (1992)[7], Vlahcevic *et al.* (1999)[8]].

Cholesterol or a 27-oxysterol (27-hydroxycholesterol or 3β-hydroxy-5-cholestenoic acid; see Figure 1-4 for chemical structures) enters the bile acid biosynthetic pathway in the endoplasmic reticulum *via* hydroxylation at position 7. These hydroxylations are performed by cholesterol 7α-hydroxylase (cyp7a) or oxysterol 7α-hydroxylase (cyp7b), respectively (Figure 1-4 reaction 1). The first step in the epimerization then occurs with production of a ketone from the 3β-hydroxyl group by 3β-hydroxy-Δ^5C_{27}-steroid oxidoreductase (reaction 2). The majority of these ketone intermediates are also hydroxylated at position 12 by sterol 12α-hydroxylase (cyp8b1; reaction 3) to produce 7α, 12α-dihydroxy-4-cholesten-3-ones which then exit the endoplasmic reticulum. In the cytosol,

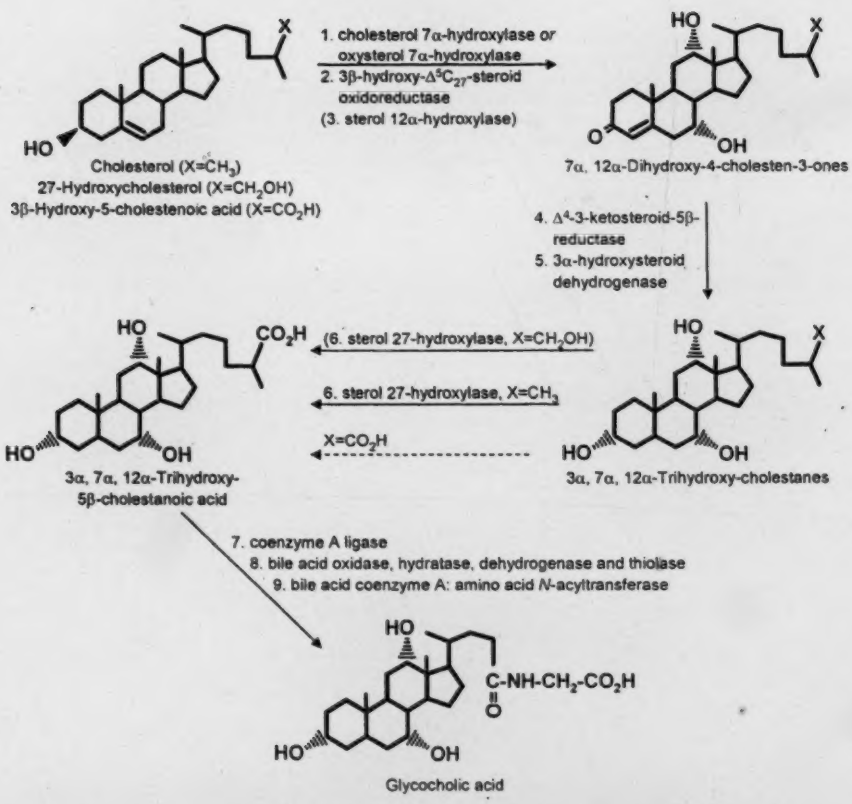

Figure 1-4. BILE ACID BIOSYNTHESIS. A, Bile acid synthesis begins with 7α-hydroxylation (reaction 1) of either cholesterol ($X=CH_3$) or oxysterols such as 27-hydroxycholesterol ($X=CH_2OH$) or 3β-hydroxy-5-cholestenoic acid ($X=CO_2H$). Reactions 2-5 complete the hydroxylation and saturation of the steroid ring. Hydroxylation at position 12 (reaction 3) is optional as indicated by the parentheses. Side chain modification begins with oxidation at position 27 (reaction 6) to form 3β-hydroxy-5-cholestenoic acid. If $X= CH_2OH$, oxidation may also be performed by alcohol and aldehyde dehydrogenases (as indicated by the parentheses). The dashed arrow indicates that no enzymatic conversion is required if $X=CO_2H$. Truncation of the side chain is completed via reactions 7 and 8. Amino acid conjugation (reaction 9) completes bile acid synthesis.

steroid ring saturation is catalyzed by Δ^4-3-ketosteroid-5β-reductase (reaction 4) and epimerization is completed with the reformation of a hydroxyl group at position 3. However, this hydroxyl group is now axial in orientation (the same as the 7α- and 12α- hydroxyl groups but opposite to the 3β-hydroxyl group of cholesterol). The stereospecific production of the 3α-hydroxyl is a defining

characteristic of all bile acids and is catalyzed by 3α-hydroxysteroid dehydrogenase (reaction 5). This reaction also marks the completion of the steroid ring modifications and movement of these 3α, 7α, 12α-trihydroxy-cholestanes to the endoplasmic reticulum for processing of the side chain.

The remaining steps in bile acid biosynthesis all facilitate truncation of the cholesterol side chain by three carbons. The first step in this process is the production of 3α, 7α, 12α-trihydroxy-5β-cholestanoic acid from intermediates lacking a 27-carboxylic acid group (thus, intermediates derived from 3β-hydroxy-5-cholestenoic acid require no further processing). Intermediates derived from cholesterol must be transported to the mitochondria where 27-hydroxylation is catalyzed by sterol 27-hydroxylase (cyp27; reaction 6). Subsequent oxidation to 3α, 7α, 12α-trihydroxy-5β-cholestanoic acid may also be catalyzed by cyp27 or by cytosolic alcohol and aldehyde dehydrogenases. In contrast, intermediates derived from 27-hydroxycholesterol need not be transported to the mitochondria and can be oxidized to 3α, 7α, 12α-trihydroxy-5β-cholestanoic acid by the cytosolic alcohol and aldehyde dehydrogenases (indicated by parentheses around reaction 6).

After all bile acid intermediates have been converted to 3α, 7α, 12α-trihydroxy-5β-cholestanoic acid, transport back to the endoplasmic reticulum is required. Here, bile acid coenzyme A ligase esterifies coenzyme A to the 27-carboxyl group (reaction 7). This intermediate is then transported to peroxisomes where bile acid oxidase introduces a double bond at position at 24. The dual-function enzyme bile acid hydratase/dehydrogenase hydrates this double bond

and hydroxylates position 24. Bile acid thiolase then catalyzes the release of propionate ($CH_3CH_2CO_2H$) thereby completing side chain truncation (reaction 8). Finally, this coenzyme A-bile acid is conjugated to glycine or taurine by bile acid CoA:amino acid *N*-acyltransferase (reaction 9).

1.4.2 Subcellular enzyme locations – The conversion of cholesterol and oxysterol substrates to bile acids is represented as a linear sequence of events in Figure 1-4. However, the 27-hydroxycholesterol or 3β-hydroxy-5-cholestenoic acid substrates are produced from cholesterol by cyp27. Thus, cholesterol is the lone substrate for bile acid synthesis. From cholesterol, we can describe two pathways for bile acid biosynthesis which differ in two important ways, i) the timing of cyp27 action and ii) the enzyme used for 7α-hydroxylation[7,8].

The neutral pathway (Figure 1-5A) typically accounts for 70-95% of total of bile acid biosynthesis[9] and begins with 7α-hydroxylation of cholesterol by cyp7a in the endoplasmic reticulum. Additional modifications to the steroid nucleus are completed in the endoplasmic reticulum and cytosol prior to any modifications of the side chain (reactions 1-5). Side-chain modifications begin in the mitochondria through the action of cyp27 (reaction 6) and are completed in the endoplasmic reticulum and peroxisomes (reactions 7-9). In contrast, the acidic pathway (Figure 1-5B) begins in the mitochondria with cyp27 to produce 27-hydroxycholesterol or 3β-hydroxy-5-cholestenoic acid (reaction 6; the acidic pathway derives its name from the initial production of carboxylic acids). These oxysterols move to the endoplasmic reticulum and cytosol for modification of the

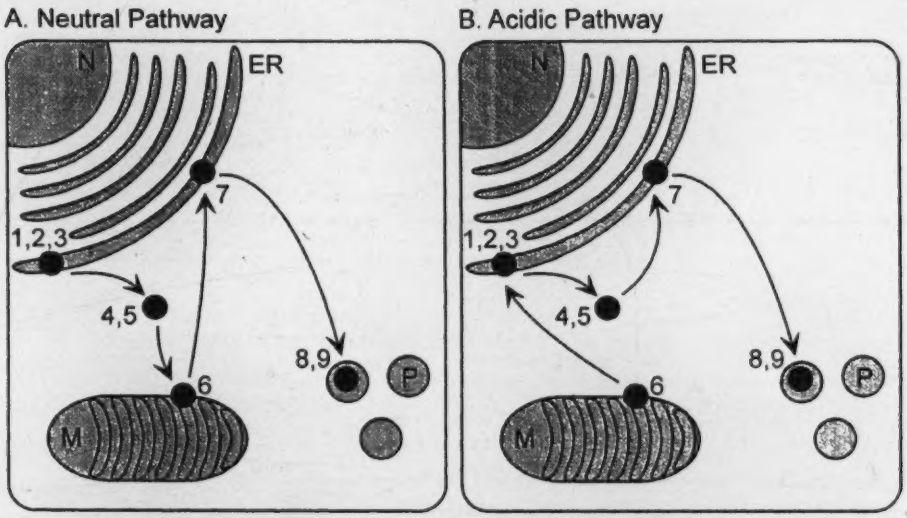

Figure 1-5. BILE ACID SYNTHESIS OCCURS IN MULTIPLE SUBCELLULAR COMPARTMENTS. The reactions required for bile acid synthesis (indicated by numerals; see Figure 1-4 for complete descriptions) occur in different organelles (indicated with shading). Bile acid intermediates (filled circle) must be transported between these organelles (indicated with arrows). ***A,*** The neutral pathway begins with cholesterol in the liver. Modifications to the steroid nucleus (1-5) are completed in the endoplasmic reticulum (ER) and cytoplasm. Side chain modifications (7-9) are completed in the mitochondria (M) and peroxisomes (P). ***B,*** The acidic pathway begins in the mitochondria with oxidation of cholesterol at position 27 (6). These oxysterols (27-hydroxycholesterol or 3□-hydroxy-5-cholestenoic acid) then proceed to the endoplasmic reticulum and cytosol where modifications to the steroid nucleus occur (1-5). The remaining side chain modifications (7-9) are identical to the neutral pathway. Cytoplasmic alcohol and aldehyde dehydrogenases, which can also convert 27-hydroxycholesterol to 3β-hydroxy-5-cholestenoic acid (reaction 6), are not shown. The nucleus (N) is shown for reference.

steroid nucleus (reactions 1-5). Side-chain modification is completed as described for the neutral pathway (reactions 7-9). Cyp27 need not be involved at this point in the acidic pathway due to the presence of cytosolic alcohol and aldehyde dehydrogenases as described above.

It is important to note a number of details of bile acid biosynthesis that have been omitted in the above descriptions for clarity. For instance, cyp7a expression is limited to hepatocytes whereas cyp27 is found in many tissues. Thus, the acidic pathway utilizes 27-oxysterols produced both in the liver and in

peripheral tissues. These oxysterols must be transported to the liver *via* the circulatory system prior to 7α-hydroxylation by cyp7b. Similarly, 25-hydroxycholesterol is a substrate for cyp7b and is synthesized at low levels in a variety of tissues, thereby necessitating its transport to the liver. Cyp7b is very specific for 25- and 27-hydroxycholesterol. In contrast, recent studies show that recombinant cyp7a has comparable 7α-hydroxylase activity towards cholesterol and 20(*S*)-, 25- or 27-hydroxycholesterol[10], indicating overlap between the neutral and acidic pathways.

In addition to the neutral and acidic pathways, additional pathways of bile acid biosynthesis are likely to exist [reviewed in Bjorkhem and Eggersten (2001)[11]]. 24(*S*)-hydroxycholesterol, the main catabolic product of cholesterol in the brain, is transported to the liver where 24-hydroxycholesterol 7α-hydroxylase (cyp39) initiates bile acid synthesis. Cyp39 may define a third bile acid biosynthetic pathway. However, recent studies show that cyp7a may also catabolize 24-hydroxycholesterol, raising the possibility that cyp39 may not be required for this pathway. Further, the amount of bile acids derived from 24(*S*)-hydroxycholesterol has yet to be reported and, while important for cholesterol catabolism in the brain, this pathway may be quantitatively minor compared to bile acid synthesis *via* the neutral and acidic pathways. Another poorly understood pathway is responsible for the production of TMCA in rodents. The enzymatic activities required to produce the 6β, 7β configuration of hydroxyl groups (see Table 1-1) are currently unknown. As TMCA synthesis *in vivo* does

not require intestinal bacteria[12], identification of the enzymes involved may also define a new pathway of bile acid biosynthesis.

1.4.3 Modulators of key enzymes in bile acid biosynthesis – Regulating the amount of bile acids synthesized typically occurs by regulation of cyp7a and cyp7b, the enzymes responsible for 7α-hydroxylation in the acidic and neutral pathways. As the majority of bile acids are usually synthesized by the neutral pathway, cyp7a regulation has been characterized in great detail. However, the ability of the acidic pathway to compensate for a lack of the neutral pathway in cyp7a-null mice[13], has sparked renewed interest in cyp7b. In addition, the regulation of the ratio of CA-derived- to CDCA-derived bile acids has also experienced a resurgence of scientific interest focused primarily on cyp8b1 (which is required for 12α-hydroxylation and is likely the primary determinant of this ratio). The regulation of these enzymes will be discussed below.

1.4.3(i) Cholesterol 7α-hydroxylase – Cyp7a is the rate-limiting enzyme in the neutral pathway and is thus responsible for the majority of bile acid biosynthesis. Thus, understanding the regulatory mechanisms governing cyp7a activity has been one of the main areas of research in the filed of bile acids. Interest in understanding and manipulating cyp7a regulation has been intensified by the observation that over-expression of cyp7a reduces plasma cholesterol concentrations[14,15] and protects against atherosclerosis in mice[16]. Due to the large volume of information on cyp7a regulation, the following section is limited to

a description of the regulatory mechanisms in rodents. Three categories are discussed: dietary modulators, hormonal modulators and post-translational regulation. Regulatory mechanisms in non-rodent species are discussed thereafter in a separate section and comparisons to rodents are presented.

Dietary Modulators (Bile Acids)

Perhaps the most consistent aspect of cyp7a regulation is the feedback inhibition of hydrophobic bile acids on cyp7a activity. The manipulation of the bile acid pool size by administration of bile acids or bile acid binding resins has demonstrated this effect convincingly in rats[17], mice[18], hamsters[19] and guinea pigs[20]. The cloning of the cholesterol 7α-hydroxylase gene (*Cyp7a1*) permitted further analysis of relative cyp7a mRNA levels by RNA blotting and ribonuclease protection assays. These analyses revealed that changes in cyp7a activity were tightly correlated with cyp7a mRNA abundance[21-23]. Additional experiments in rodent hepatocytes and rodent-derived cell lines (examining both the endogenous cyp7a mRNA as well as the activity of transfected cyp7a1 promoter-reporter gene chimeras) provided further support for the ability of bile acids to reduce cyp7a mRNA levels[24-28] and gave rise to the notion that cyp7a regulation in rodents was primarily transcriptional.

Although feedback repression of cyp7a activity and mRNA levels has been documented in the literature more often than any other effector of cyp7a, the mechanism of this repression remained a mystery for some time. In 1995, Stravitz *et al.* showed that protein kinase inhibitors interfered with bile acid-

mediated repression of cyp7a mRNA levels[26]. Although this implied the involvement of signal transduction pathways and was supported by previous reports that cyp7a activity could be mediated *in vitro* by phosphorylation/ dephosphorylation (see below), the details of the signaling pathway(s) were unknown. Other investigators approached the problem of solving the mechanism from a different angle: rather than start at the stimuli and progress to the gene, they started at the gene and began to work towards a number of possible stimuli. In other words, the research focus shifted to characterizing the transcription factors bound to the *Cyp7a1* gene promoter [reviewed in Repa and Mangelsdorf (2000)[29]] and then identifying the stimuli mediated by these proteins. This approach had been successfully applied to identify the receptors required for the regulation of cyp7a by cholesterol (see below) and in 1999, two groups independently described the farnesoid X receptor (FXR), a member of the nuclear hormone receptor superfamily[i], as a candidate for transcriptional regulation by bile acids[30,31].

Although FXR had been first characterized by its ability to bind farnesoids, it bound bile acids with a much higher affinity. Also, the binding of these bile acids activated the transcription of promoter-reporter gene chimeras containing FXR response elements (FXREs[ii]) in cultured cells[30-32]. However, the fact that nuclear receptors typically activate gene expression in response to ligand binding raised the question of how bile acids might stimulate FXR and yet repress

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[i] Nuclear hormone receptors typically exist in the nucleus bound to DNA. In this state, they interact with corepressors and repress gene transcription. The binding of the receptor ligand causes a dissociation of the corepressor, recruitment of a coactivator and increased transcription.

[ii] A typical FXRE consists of two hexanucleotide motifs (related to the sequence 5'-AGGTCA-3') arranged as direct repeats separated by 1 nt (DR+1).

Cyp7a1 gene expression. The debate was further fueled by the demonstration that FXR could inhibit the activity of a cyp7a1 promoter-reporter gene chimera without binding to the *Cyp7a1* gene promoter[33]. The solution came less than a year later when Goodwin *et al.*[34] and Lu *et al.*[35] independently reported the regulatory cascade leading to the bile acid-mediated repression of cyp7a. As shown in Figure 1-6, two other transcription factors, the liver receptor homolog 1 (LRH-1) and the small heterodimeric partner (SHP), are involved in this cascade. LRH-1 is required for the liver-specific expression of the *Cyp7a1* gene and binds as a monomer to a conserved sequence in the *Cyp7a1* gene promoter (LRHRE). SHP does not bind DNA directly but rather interacts with LRH-1 and interferes with its ability to activate gene transcription. When FXR is activated by bile acid ligands, it binds to the FXRE in the *SHP* gene promoter and stimulates production of SHP protein. This in turn binds to LRH-1 at the *Cyp7a1* gene promoter causing a reduction in transcription. Deactivation of FXR due to a reduced bile acid pool size causes a reduction in *SHP* gene expression/SHP protein and LRH-1 is able to function as a transcriptional activator.

The coordinated effects of FXR, LRH-1 and SHP explain many of the effects of bile acids on *Cyp7a1* gene expression in rodents and rodent-derived cell lines. However, this model does not explain the involvement of signal transduction pathways as noted above. In addition, dietary cholic acid is still able to repress cyp7a mRNA abundance by approximately 60% in *FXR*-null mice[36] suggesting additional mechanisms of bile acid-mediated repression of gene expression. Gupta *et al.*[37] recently reported that the *SHP* gene promoter can be

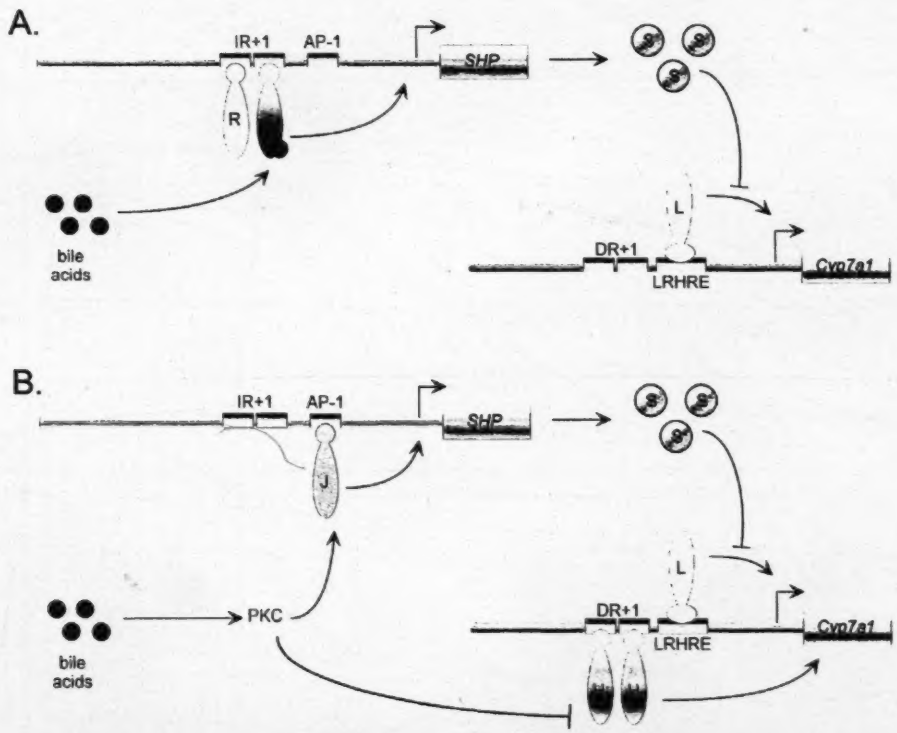

Figure 1-6. MECHANISMS OF BILE ACID-MEDIATED TRANSCRIPTIONAL REPRESSION OF THE *CYP7A1* GENE. ***A,*** Bile acids are ligands for the farnesoid X receptor (F) which binds as a heterodimer with the retinoid X receptor (R) to the IR+1 sequence in the small heterodimeric partner (*SHP*) gene promoter to induce gene expression and SHP protein (S) production. SHP then binds to liver receptor homolog 1 (L), which itself is bound to a LRH-1 response element (LRHRE) in the *Cyp7a1* gene promoter, and prevents activation of *Cyp7a1* gene expression. ***B,*** Bile acids also activate the protein kinase C (PKC) pathway. *Cyp7a1* gene expression is repressed by i) inducing *SHP* gene expression (via the binding of activated c-Jun (J) to the AP-1 sequence in the *SHP* gene promoter) and ii) by dampening the activation potential of hepatocyte nuclear factor 4 (H) bound as a homodimer to the DR+1 sequence of the *Cyp7a1* promoter.

stimulated by TCA-induced signal transduction pathways. De Fabiani *et al.*[38] have shown that the activation potential of hepatocyte nuclear factor 4 (HNF-4), which binds to a sequence adjacent to the LRHRE in the *Cyp7a1* gene promoter and stimulates *Cyp7a1* gene transcription, can be dampened by CDCA-induced signal transduction pathways. Although the magnitude of the response *via* these mechanisms is typically modest compared to the FXR/LRH-1/SHP pathway, they

provide another level of regulation which may be sensitive to a broader range of bile acids or may be able to change *Cyp7a1* gene expression at a different range of bile acid concentrations.

Dietary Modulators (Cholesterol)

In a classic feed-forward activation pathway, dietary (exogenous) cholesterol and oxysterols increase cyp7a enzyme activity in mice[18], rats[20,39,40] and rat hepatocytes[41]. A parallel increase in cyp7a mRNA abundance was also observed in mice and rats[18,22,23,42] which reinforced the importance of transcriptional regulation of cyp7a in rodents. Furthermore, pharmacological inhibition of *de novo* cholesterol synthesis resulted in reduced cyp7a activity and mRNA abundance in rats[43] and rat hepatocytes[41], suggesting that endogenous cholesterol was also important in regulating cyp7a. The mechanism of regulation by cholesterol was discovered by Lehmann *et al.*[44] in 1997, three years prior to the reports of the FXR/LRH-1/SHP cascade. This laboratory demonstrated that two liver X receptor (LXR) isoforms (LXRα and LXRβ) bound to a direct hexanucleotide repeat separated by 4 nt (DR+4) downstream of the LRHRE in the *Cyp7a1* gene promoter[44]. They also demonstrated that a number of natural oxysterols, but not cholesterol itself, activated these LXRs resulting in increased promoter activity. The inability of cholesterol to directly activate LXR seemed paradoxical. However, *in vivo* studies supported the role of LXR in regulating cyp7a. Administration of non-steroidal LXR agonists to mice resulted in increased cyp7a mRNA abundance[45] while dietary cholesterol failed to significantly

increase cyp7a mRNA levels in *LXRα*-null mice[46]. Thus, the current model of cyp7a regulation by cholesterol (Figure 1-7) requires the conversion of dietary cholesterol to oxysterols by the variety of cholesterol hydroxylases expressed throughout the body [reviewed in Russell (2000)[47]]. These oxysterols then activate LXR bound to the *Cyp7a1* gene promoter and induce gene transcription. The ability of 24(*S*), 25-epoxycholesterol (an intermediate in *de novo* cholesterol biosynthesis[48,49]) to activate LXR suggests that increased cholesterol biosynthesis can also feed-forward to activate cyp7a and explains, at least in part, the ability of statins to repress cyp7a[43].

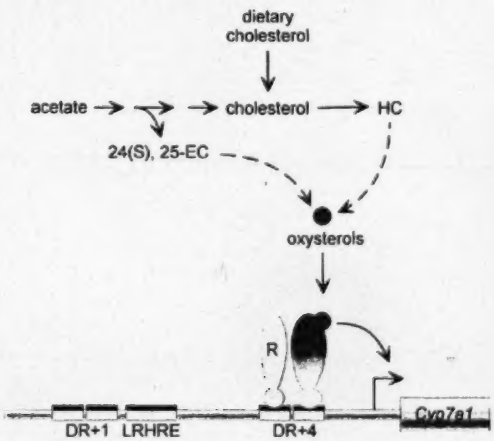

Figure 1-7. REGULATION OF CYP7A1 GENE EXPRESSION BY OXYSTEROLS. The liver X receptor (LX) binds as a heterodimer with the retinoid X receptor (R) to the DR+4 sequence of the *Cyp7a1* gene promoter. 24(*S*), 25-epoxycholesterol (EC), produced during *de novo* cholesterol synthesis from acetate, and 24(*S*)-hydroxycholesterol (HC), produced from endogenous and dietary cholesterol, move to the nucleus (indicated by dashed arrows) and activate LXR. Activated LXR increases *Cyp7a1* gene transcription. The relative locations of the DR+1 and LRHRE sequences (see Figure 1-6) are also shown.

DIETARY MODULATORS (FATTY ACIDS)

Sources of dietary cholesterol often contain significant amounts of other lipids such as triacylglycerol and phospholipids. Thus, the ability of these lipids, to regulate cyp7a has been studied to some degree. Fatty acids, produced from both triacylglycerol and phospholipids during digestion, appear to regulate cyp7a

but the current data in the literature is inconsistent. The role of fatty acids is further confounded by the use of semi-purified diets from different sources or purified fatty acids in the presence of cholesterol. However, these reports together show that the species of fatty acid present is critical in determining the response of cyp7a. For example, Kurushima *et al.*[50] demonstrated that, in the presence of 0.1% cholesterol, oleic acid (18:0) increased cyp7a activity in hamsters while palmitic acid (16:0) had no effect. Cheema and Agellon[51] showed that cyp7a activity is lower in mice fed a semi-purified diet enriched in saturated fatty acids (18:0) compared to mice fed a diet enriched in either mono- or polyunsaturated fatty acids (18:1, and 18:2(n-6), respectively). In addition, they also showed that the stimulatory effect of cholesterol on cyp7a activity and mRNA abundance could be reversed by diets enriched with monounsaturated and saturated fatty acids[51,52] suggesting interplay between fatty acid and sterol regulation of cyp7a.

Like bile acids and oxysterols, the effects of fatty acids on cyp7a are likely to be mediated by a nuclear hormone receptor. The alpha isoform of the peroxisome proliferator activated receptor (PPARα) is the most likely candidate receptor as it is activated by fatty acids (and fibrates) and is abundant in the liver[53]. However, the mechanism of action is unclear. There is some controversy in the literature as to if and where PPARα might bind the rodent *Cyp7a1* gene promoter. Conflicting reports as to the activation or repression of cyp7a1 promoter-reporter gene chimera activity[54,55] further cloud the interpretation of the available data. However, Princen *et al.* has demonstrated that fibrates repress

cyp7a activity and mRNA abundance in wild type mice but not in PPARα-null mice[56] suggesting a role for PPARα in cyp7a regulation. More research is required to determine whether fibrates and fatty acids have similar effects on cyp7a. In addition, it is important to determine the purpose of the induction or repression of cyp7a by fatty acids.

Dietary Modulators (Various)

Dietary fiber appears to stimulate cyp7a activity and *cyp7a mRNA* abundance[57-59]. In mice, these effects are correlated with increased bile acid excretion[59] suggesting that the stimulation of cyp7a is due to the loss of repression by bile acids. Cafestol, a chemical present in boiled coffee, simultaneously raises plasma cholesterol and reduces cyp7a activity[60]. The mechanism is unknown but the structural similarity between cafestol and cholesterol suggests antagonism of LXR-oxysterol transactivation of *Cyp7a1* gene expression. Finally, dietary taurine, found readily in milk, eggs, fish and red meat, stimulates cyp7a activity in rats[61]. This effect seems appropriate as these foods can also contain high levels of cholesterol. It may also indicate that bile acid synthesis *via* cyp7a is a means of excretion not only for cholesterol, but also for taurine.

Hormonal Modulators (Glucocorticoids)

Glucocorticoid hormones are secreted from the adrenals in response to stress/stimuli and display a diurnal rhythm. The observation that increased

glucocorticoids were correlated with increased cyp7a activity[62] was the first evidence of a link between these hormones and bile acid synthesis. Subsequent studies revealed that cyp7a activity, protein mass and cyp7a mRNA abundance were tightly correlated in rats during this diurnal rhythm[63,64]. In addition, dexamethasone (dex), a synthetic glucocorticoid, induced bile acid synthesis and cyp7a activity in primary rat hepatocytes[65]. These changes were blocked in the presence of protein and RNA synthesis inhibitors[65]. Together, these data suggested that glucocorticoids regulated cyp7a *via* transcriptional mechanisms. Indeed, functional characterization of cyp7a1 promoter-reporter gene chimeras revealed that the ability of dex to stimulate promoter activity in HepG2 cells (a human liver-derived immortalized cell line) was dependent upon i) a sequence within 300 nt of the transcription start site and ii) the cotransfection of an expression vector encoding the glucocorticoid receptor (GR)[66,67]. However, a direct interaction between the GR and the *Cyp7a1* gene promoter has never been reported.

Several lines of evidence suggest that glucocorticoids stimulate *Cyp7a1* gene expression and cyp7a activity through indirect mechanisms. Mitropoulos *et al.*, demonstrated that, in the absence of glucocorticoids (achieved *via* adrenalectomy), the diurnal rhythm of cyp7a and bile acid synthesis was still present, but the magnitude of the change was blunted[68]. They also showed that cortisol (a natural glucocorticoid) injection three hours prior before the acrophase (point of peak activity) of cyp7a activity restored the diurnal increase in cyp7a activity to normal levels. Importantly, cortisol injection three hours before the

nadir (point of minimum activity) of cyp7a activity did not affect enzyme activity and strongly suggests that another protein is involved in glucocorticoid regulation.

A comparison of the acrophase for plasma cortisol concentrations and cyp7a activity may also suggest the involvement of a second protein in the diurnal rhythm of cyp7a. As shown in (Figure 1-8), cortisol levels[69] peak at least 3 hours before the maximum cyp7a activities[70] are observed. In contrast, the acrophase of albumin site D-binding protein (DBP) mRNA levels[71,72] is closer to the acrophase of cyp7a activity. Expression of the *DBP* gene is enriched in the liver and displays a diurnal rhythm[73]. The ability of this transcription factor to bind and transactivate the *Cyp7a1* gene promoter has been well characterized. DBP binds the *Cyp7a1* gene promoter at a number of locations *in vitro* but the FP-2 sequence, centered at -252 nt, appears to be most important for DBP-mediated

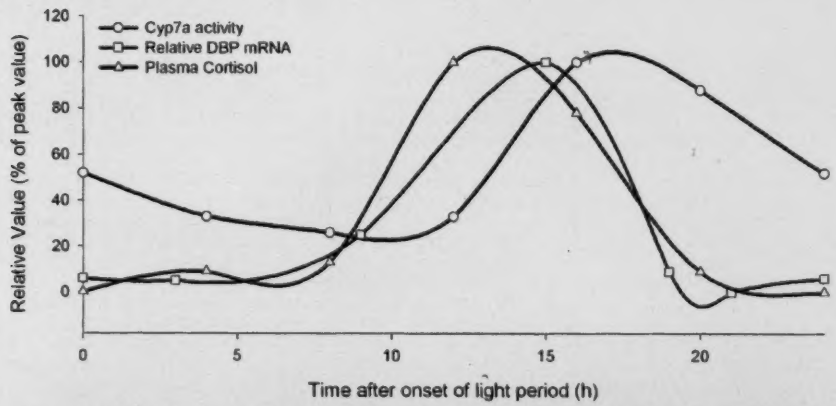

Figure 1-8. CIRCADIAN RHYTHM OF CYP7A ACTIVITY, DBP MRNA ABUNDANCE, AND PLASMA CORTISOL IN RODENTS. The circadian rhythm of cyp7a activity, DBP mRNA abundance, and plasma cortisol were determined in rodents maintained *ad libitum* on standard chow diets under a 12 hour light-dark regimen (see text for references). The maximum value measured in each parameter was taken as 100% and plotted as a function of time after the onset of the light period (i.e. lights on at 0h, off at 12h).

transactivation[74-76] and is in the region identified previously as important for glucocorticoid regulation. Glucocorticoids regulate the expression of a number of genes that are expressed with diurnal rhythm, including the *DBP* gene. Thus, it is attractive to postulate that glucocorticoids regulate cyp7a indirectly *via* DBP.

The link between glucocorticoids, DBP and cyp7a has not been completely elucidated but the increased understanding of the regulation of circadian clocks in eukaryotes has provided a number of insights. In mammals, circadian rhythms appear to be controlled by a master pacemaker in the central nervous system and slave oscillators in most peripheral tissues [reviewed in Ripperger and Schibler (2001)[77]] and shown in Figure 1-9). As the name suggests, the circadian clock can only approximate a 24 hour schedule and is reset each day by light stimuli. Light stimulates the retina which in turn resets the master pacemaker in the hypothalamus. One mechanism whereby the master pacemaker resets the peripheral slave oscillators is to stimulate the release of glucocorticoids[iii] into the circulatory system. These hormones travel to the periphery and exert their 'reset' functions throughout the body[78]. The *DBP* gene responds to this stimulus and may thus transduce the glucocorticoid signal to the *Cyp7a1* gene as described above.

It should be noted that glucocorticoids are not the only way in which the master pacemaker communicates with the periphery. As described above, cyp7a activity in adrenalectomized rats still displays a diurnal pattern, as does *DBP* gene expression[69]. The inability of exogenous cortisol to stimulate cyp7a when

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[iii] The hypothalamus stimulates the pituitary to release adrenocorticotropic hormone. This travels *via* the circulation to the adrenals and stimulates glucocorticoid secretion.

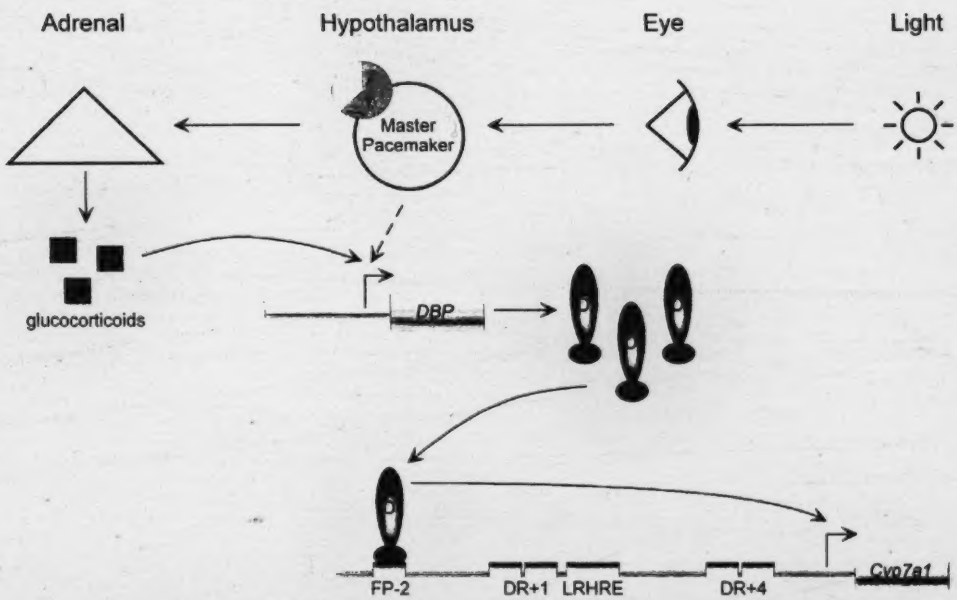

Figure 1-9. PUTATIVE MECHANISM OF GLUCOCORTICOID/DBP-MEDIATED TRANSACTIVATION OF THE *CYP7A1* GENE PROMOTER. External time cues reset the master pacemaker in the hypothalamus via the retina of the eye and the retinohypothalamic tract. The master pacemaker can then reset the slave oscillators in peripheral tissues such as the liver by stimulating a burst of glucocorticoid secretion from the adrenals (via the pituitary, not shown) as well as other uncharacterized mechanisms (indicated by the dashed arrow). In the liver, *DBP* gene expression is induced by the sudden rise in plasma glucocorticoids. DBP protein (D) binds to the FP-2 sequence in the *Cyp7a1* gene promoter and induces transcription in the absence of ligand. The location of the FP-2 sequence relative to DR+1, LRHRE and DR+4 is indicated.

injected before the nadir of cyp7a activity can thus be explained by a lack of DBP to transduce the glucocorticoid signal to the *Cyp7a1* gene promoter[iv]. Finally, glucocorticoids can also induce circadian *DBP* gene expression in cultured cells[78], providing a possible explanation for the ability of dex to stimulate the activity of the *Cyp7a1* gene promoter in HepG2 cells (above).

[iv] DBP gene expression maintains a diurnal rhythm following adrenalectomy, and would not be present during the nadir of cyp7a activity

HORMONAL MODULATORS (THYROID HORMONE)

Another class of hormonal regulators of cyp7a are the thyroid hormones (see Section 1.5 for a description of thyroid hormone metabolism). The ability of thyroid hormones to induce (or thyroidectomy to reduce) cyp7a activity in rodents is well documented[79-84] and is correlated with mRNA abundance and *Cyp7a1* gene transcription in hypophysectomized rats[v85-87], as well as intact mice[88]. Many of the effects of thyroid hormones are mediated by thyroid hormone receptors (TRs) which are also members of the nuclear hormone receptor superfamily. Gullberg *et al.* showed that *TRβ*-null mice, but not *TRα*-null mice, failed to display thyroid hormone-induced increases of cyp7a mRNA abundance[88]. This data strongly implicates TRβ in mediating the effects of thyroid hormone on cyp7a. However, thyroid hormones do not affect cyp7a activity or mRNA abundance in primary rat hepatocytes[89,90] nor do they affect the activity of cyp7a1 promoter-reporter gene chimeras in HepG2 cells[66]. The lack of an *in vitro* model system to study T_3 regulation has precluded the discovery of TR binding sequences in the *Cyp7a1* gene promoter. These observations raise the possibility that thyroid hormones mediate their effects on cyp7a *via* an indirect mechanism present in intact rodents.

A mechanism that may explain the inability of thyroid hormone to stimulate cyp7a in cultured cells is suggested by the apparent interaction between thyroid hormone and glucocorticoids. In isolated rat hepatocytes, thyroid hormone does not affect cyp7a mRNA abundance while dex causes a two-fold increase.

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[v] Hypophysectomy is the surgical ablation of the pituitary. This procedure disrupts endocrine signaling between the pituitary and endocrine glands, effectively preventing glucocorticoid and thyroid hormone secretion.

Together, thyroid hormone and dex act synergistically to increase cyp7a mRNA abundance five- to twenty-fold[89,90]. If the effects of dex on cyp7a are actually mediated by DBP (above), thyroid hormone may potentiate the ability of glucocorticoids to induce *DBP* gene expression in these cultured cells. *In vivo*, thyroid hormones display a circadian rhythm and are able to induce *Cyp7a1* gene expression in the absence of glucocorticoids[85-87]. It is possible that thyroid hormones may be another means of communication between the master pacemaker in the hypothalamus and the slave oscillator(s) in the liver and thus regulate cyp7a *via* DBP. Although there is no direct evidence to support this mechanism, the dampened diurnal rhythm of cyp7a activity[68] and DBP protein levels[69] following adrenalectomy is consistent with this concept. Further support is provided by Pandak *et al.* who compared the effects of endocrine organ ablation on cyp7a in rats. The effect of hypophysectomy on cyp7a mRNA abundance and *Cyp7a1* gene transcription is greater than the combined effects of adrenalectomy and thyroidectomy[83], consistent with synergism between glucocorticoids and thyroid hormone. More research is clearly required to understand the mechanism of thyroid hormone-induction of cyp7a.

Hormonal Modulators (Various)

Growth hormone stimulates cyp7a activity and bile acid excretion in rats[91]. However, the increased activity is not correlated with cyp7a mRNA abundance in mice[92] suggesting that post-transcriptional mechanisms are required for regulation by growth hormone. Estrogen, when administered at physiological

levels, can also increase cyp7a activity, cyp7a mRNA abundance and bile acid synthesis in both rats and rat hepatocytes[93,94] while insulin appears to have an opposite effect on these parameters[95,96]. A sequence in the proximal rat and hamster *Cyp7a1* gene promoters is essential for the effect of insulin in a hepatocyte-derived cell line[66,67]. However, the molecular details of this response are not known.

Post-translational Regulation

The regulation of cyp7a appears to occur primarily at the level of gene expression. However, cyp7a activity can be altered *in vitro* by manipulations such as incubation with phosphatase inhibitors or phosphatases which increase or decrease the phosphorylation state of the enzyme, respectively[97-103]. Under these circumstances, cyp7a activity is higher when phosphorylated and reduced when dephosphorylated. However, the *in vivo* consequences of these putative phosphorylation states are not known. It has been proposed that regulating the phosphorylation status of cyp7a may be a short-term method of controlling enzyme activity in response to dietary/hormonal changes[104]. Indeed, there are numerous examples in the literature where cyp7a activity and cyp7a mRNA levels do not correlate[23,40,51,92,105,106]. These differences may be explained by alterations in the pattern of cyp7a phosphorylation. However, a careful examination of potential phosphorylation sites in cyp7a has not been reported. Further, most of the modern experiments in the cyp7a field (i.e. since the cloning of the rat gene in 1990[42]) examine the effects of chronic exposure to a stimulus

and are thus less likely to observe these short-term changes in cyp7a activity. Thus, the role of phosphorylation-dephosphorylation in regulating cyp7a activity *in vivo* is currently unknown.

SPECIES-SPECIFIC DIFFERENCES IN CYP7A REGULATION

As many of the regulatory mechanisms governing cyp7a activity involve the binding of transcription factors to the gene promoter, a comparison of promoter sequences can provide valuable information. Figure 1-10 shows selected sequences from the *Cyp7a1* gene promoter of human, monkey, pig, rat, mouse and hamster[vi]. The characterized regulatory sequences described above are indicated in addition to a number of putative regulatory sequences related to the consensus nuclear hormone receptor binding sequence 5'-AGGTCA-3'. Sequences in close proximity have been grouped and are designated Site I, Site II, and Site III based on their relative distance from the transcription start site. The sequence of Site I, Site II and Site III will be discussed in regard to the regulation of cyp7a in various species.

A number of the mediators of cyp7a in rodents are also able to regulate cyp7a in other species. Feedback inhibition of cyp7a activity and mRNA abundance by bile acids or loss of inhibition due to cholestyramine[vii] administration or bile drainage is also observed in rabbits[20,107-110], humans[111-115]

[vi] Promoter sequences (except monkey) were obtained from GenBank™/EBI Data Bank using the following accession numbers: human (L20569), pig (AF020317), rat (J02926), mouse (L23754) and hamster (L04690). The promoter sequence from African green monkey was obtained from Dr. L. Rudel (personal communication).

[vii] Cholestyramine is a bile acid binding resin which reduces the bile acid pool size by increasing fecal bile acid excretion.

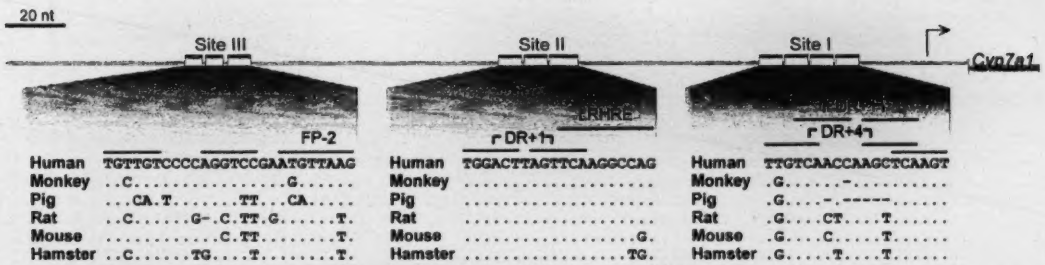

Figure 1-10. SEQUENCE ANALYSIS OF REGULATORY ELEMENTS IN THE *CYP7A1* GENE PROMOTER OF SIX SPECIES. The sequences of known and putative regulatory elements in the *Cyp7a1* gene promoter of six species are shown. Sequences in close proximity are grouped together and are designated Site I, Site II and Site III. Nucleotides identical to the human sequence are indicated with "." while sequence gaps are designated with "-". The location of known and putative regulatory elements is indicated with lines above the sequence. Previously characterized regulatory sequences are further annotated with the appropriate name above the line.

and HepG2 cells[116-118]. The conservation of the LRHRE and the DR+1 sequences in Site II (which overlap by 3 nt) suggests that the LRH/SHP cascade (see Figure 1-6) is functional in these species. The observation that the human homolog of LRH-1 binds the LRHRE in the human *CYP7A1* gene promoter *in vitro*[119] strongly supports this hypothesis.

Another conserved feature of cyp7a regulation is the circadian rhythm. A diurnal variation of cyp7a activity has been reported in rabbits[64], monkeys[120] and pigeons[121]. Analysis of the DBP binding site FP-2 (see Figure 1-9) from various rodents and primates reveals an 87.5% sequence identity (7 of 8 nt) in this region (Figure 1-10). Homologs of DBP are thus likely to bind the *Cyp7a1* gene promoters in these species and mediate the circadian rhythm. The FP-2 sequence in the porcine *Cyp7a1* gene promoter is only 62.5% identical to the rodent sequence. It is not known if DBP can bind to this sequence or if porcine cyp7a is under circadian control.

As noted above, the regulation of cyp7a by dietary fatty acids is a matter of some controversy. However, detailed promoter analysis revealed that PPARα, when heterodimerized with the retinoid X receptor (RXR), could bind the murine *Cyp7a1* gene promoter at two locations: the DR+1 of Site II and a novel DR+1 overlapping the DR+4/LXR recognition sequence at Site I[55] (see Figure 1-10). In contrast, PPARα/RXRα could not bind the Site I DR+1 in the human *CYP7A1* gene promoter as the sequence is not conserved in this species. As a result, the activity of the human promoter was only modestly transactivated by PPAR-agonists (1.5-fold) while the murine promoter activity was increased 11-fold[55]. These results raise the possibility that humans and mice respond differently to dietary fatty acids *in vivo*. In addition, competition between LXR and PPAR binding at Site I may allow crosstalk to occur between these dietary stimuli as suggested by the ability of some fatty acids to abrogate the stimulation of *Cyp7a1* gene expression by dietary cholesterol (discussed above).

Unlike bile acids, the effects of cholesterol on cyp7a vary between species. While rodent cyp7a is stimulated by cholesterol, monkeys and some strains of rabbits fed a cholesterol-enriched diet displayed reduced cyp7a activity and *cyp7a mRNA* abundance[20,120,122-124]. The sequence of the rabbit promoter is not available but the DR+4 at Site I in the monkey *Cyp7a1* gene (see Figure 1-10) has 3 nt differences and a 1 nt gap in comparison to the rat Site I DR+4. Thus, it is unlikely that the monkey *Cyp7a1* gene promoter is bound by LXR:RXR *in vivo*. Further, the human Site I DR+4 does not bind LXR:RXR[125,126] and human cyp7a1 promoter-reporter gene chimeras are not activated by LXR agonists in

HepG2 cells[125]. A lack of LXR binding at the Site I DR+4 is consistent with the inability of dietary cholesterol to stimulate cyp7a in monkeys and may also explain the similar observations in rabbits. However, the mechanism of cholesterol-mediated repression of enzyme activity and *cyp7a mRNA* abundance is unclear. In rabbits, the repression of cyp7a by cholesterol is correlated with an increased bile acid pool size[123] which presumably activates the feedback inhibition pathway (Figure 1-6). However, bile acid pool size was not measured in cholesterol-fed monkeys preventing a similar correlation in this species. The observation that cyp7a activity and bile acid synthesis in HepG2 cells is reduced by the LXR agonist 25-hydroxycholesterol[116,127] suggests that an intact EHC (and thus bile acids) is not required for cholesterol-mediated repression. The exact mechanism of cholesterol action on cyp7a in primates remains a mystery.

Another modulator of cyp7a that has been studied in some detail in non-rodent species is thyroid hormone. Wang *et al.*[128] reported that the activity of a human cyp7a1 promoter-reporter gene chimera was repressed by thyroid hormone in HepG2 cells. This finding contrasted the absence of any effect of thyroid hormone on the murine *Cyp7a1* gene promoter *in vitro* as well as the ability of thyroid hormone to increase cyp7a mRNA abundance in intact rodents (as discussed above). Using a semi-quantitative method to study cyp7a activity[129], Sauter *et al.*[130] reported that cyp7a activity in patients with hypo- and hyperthyroidism was unaffected by treatments which restored normal plasma thyroid hormone concentrations. However, the authors failed to point out that cyp7a activity in untreated hyperthyroid patients was 27% lower than in untreated

Table 1-2. CROSS-SPECIES COMPARISON OF THE EFFECTS OF DIETARY AND HORMONAL STIMULI ON THE ACTIVITY OR MRNA ABUNDANCE OF CYP7A .

	Effect on cyp7a activity or mRNA abundance		
Stimulus	Rodent	Rabbit	Primate
Bile Acid	↓	↓	↓
Cholesterol	↑	↓	↓
Fatty Acid	↑ and ↓	?	?
Circadian Rhythm	↑	↑	↑
Thyroid Hormone	↑	?	↓

The qualitative effects of various dietary and hormonal stimuli on cyp7a activity or mRNA abundance are shown (see text for references). ↑ indicates increased activity; ↓ indicates decreased activity; ↔ indicates that both increased and decreased activity have been reported; ? indicates that this stimuusi has not been tested for the indicated species.

hypothyroid patients. The ability of thyroid hormone to repress cyp7a was also demonstrated in primary human hepatocytes. A combination of thyroid hormone and dex reduced cyp7a mRNA abundance in hepatocyte preparations from 2 donor livers[90]. Together, the data suggest that thyroid hormone represses human cyp7a activity *in vivo*. However, the basis for this species-specific difference is not known.

Table 1-2 summarizes some of the species-specific differences in cyp7a regulation.

1.4.3(ii) Oxysterol 7α-hydroxylase – Although expressed in a number of tissues, oxysterol 7α-hydroxylase (cyp7b) activity is highest in the liver[131], the site of all bile acid biosynthesis. Mice lacking cyp7b accumulate 25- and 27-hydroxycholesterol in both plasma and tissue[132] while cyp7a-null mice display an increased oxysterol 7α-hydroxylase activity which is correlated with the survival of pups beyond weaning[133]. Accumulation of hydroxycholesterol intermediates has also been observed in an infant human homozygous for a premature stop

codon in the *Cyp7b1* gene which results in an inactive protein[134]. Thus, cyp7b is required for oxysterol catabolism *via* the alternate pathway and is an important enzyme during neonatal life. However, cyp7b activity in adult mice is not affected by dietary cholesterol, dietary bile acids or genetic disruption of LXR[46,135]. The *Cyp7b1* gene promoter is only 30% identical to the *Cyp7a1* gene promoter. Further, the activity of the *Cyp7b1* gene promoter is very high in HepG2 cells[131], unlike the low activity observed for the *Cyp7a1* gene promoter. While the effects of other dietary stimuli and hormones have yet to be reported, these results show that cyp7b and cyp7a regulation is very different and suggests that cyp7b is constitutive in adult animals.

1.4.3(iii) Sterol 12α-hydroxylase – Regulating the proportions of bile acids in the bile acid pool may have important physiological consequences. For instance, CA is thought to enhance lipid absorption more than CDCA but is a less potent agonist of FXR. The primary difference between the CA-derived and CDCA-derived bile acids is the 12α-hydroxyl group (see Figure 1-3 and Table 1-1). Thus, the CA:CDCA ratio is likely to be regulated by sterol 12α-hydroxylase (cyp8b). Indeed, over-expression of cyp8b in rats and rat hepatocytes significantly increases the CA:CDCA ratio without affecting total bile acid biosynthesis[136]. Sterol 12α-hydroxylase was not cloned until 1999[137]. Thus, there is limited information regarding cyp8b regulation. In rodents, hydrophobic bile acids repress cyp8b enzyme activity and mRNA abundance[138]. The mechanism of repression appears to involve the interruption of hepatocyte nuclear factor 4

(HNF4)-mediated stimulation of promoter activity by SHP[139], similar to that observed for the *Cyp7a1* gene promoter and LRH-1 (see Figure 1-6). Cholesterol and thyroid hormone also appear to repress cyp8b1 but the mechanisms involved are currently unknown[138,140].

1.5 THYROID HORMONE METABOLISM

Thyroid hormone is an important regulator of many aspects of metabolism including cholesterol and bile acid biosynthesis and catabolism. The following section briefly describes some general features of thyroid hormone structure, synthesis and function. The effects of thyroid hormone dysregulation on bile acid metabolism in humans will then be discussed.

1.5.1 Structure and synthesis – Thyroid hormones are synthesized in the thyroid gland, the largest endocrine gland in humans. The thyroid consists of two lobes situated on either side of the trachea connected by an isthmus. Within the thyroid, cells are arranged as follicles surrounded by thyroid capillaries (Figure 1-11A)[141]. These follicles are required for thyroid hormone synthesis and secretion. As illustrated in Figure 1-11B, inorganic iodide is concentrated in the follicular cell and moves passively to the follicular lumen. Iodide can then interact with thyroglobulin (TG), a 2709 amino acid glycoprotein synthesized and secreted into the lumen by follicular cells. Thyroid hormone synthesis begins with iodination of tyrosyl residues in TG. This process is catalyzed by thyroid peroxidase, a membrane-bound protein present on the apical surface of follicular

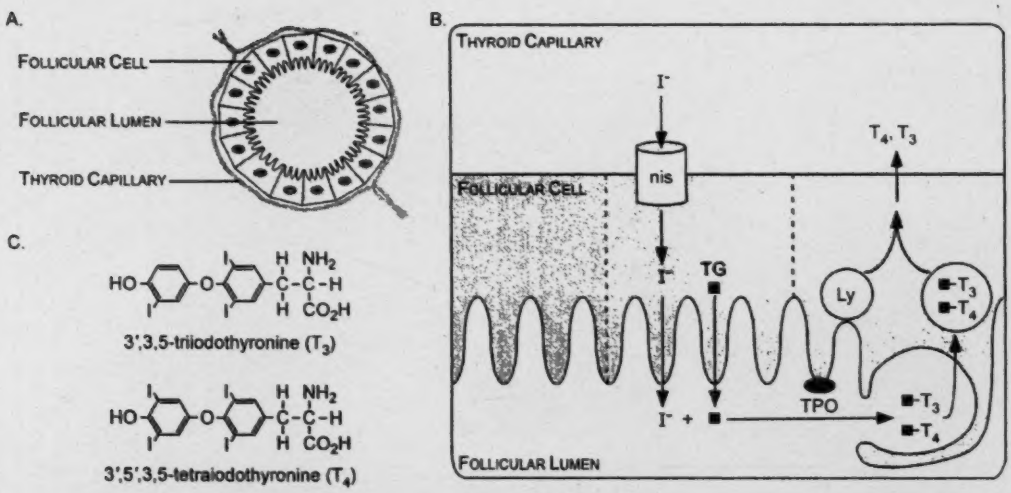

Figure 1-11. THYROID FUNCTION AND HORMONE SYNTHESIS. ***A***, The thyroid is composed of spherical follicles. The follicles are formed by the arrangement of polarized follicular cells. Each follicle is surrounded by capillaries and surrounds a mixture of glycoproteins and iodide (I^-) in the follicular lumen called the colloid. ***B***, Thyroid hormone synthesis begins with the transport of inorganic iodide into the follicular cell from the plasma via the sodium-iodide symporter (nis). Concentrated iodide then moves passively to the follicular lumen/colloid. Thyroglobulin (TG, filled square) is secreted into the colloid and is iodinated on tyrosyl residues via thyroid peroxidase (TPO) on the apical membrane of the cell. TPO further couples mono- and diiodotyrosyl residues to produce TG-bound 3',3,5-triiodothyronine (T_3) and 3',5',3,5-tetraiodothyronine (T_4). Intracellular droplets containing these protein-bound hormones are formed by the endocytosis of colloid by the follicular cells. These colloid droplets then fuse with lysosomes (Ly) and TG is proteolyzed, releasing T_3 and T_4 into the cytosol and eventually into the plasma. ***C***, The chemical structures of T_3 and T_4 are shown.

cells. Thyroid peroxidase also catalyzes the coupling of mono- and diiodotyrosyl residues to produce TG-bound 3',3,5,-triiodothyronine (T_3) and 3',5',3,5,-tetraiodothyronine (T_4), the two types of thyroid hormones (Figure 1-11C). These TG-bound hormones are endocytosed as vesicles by the follicular cells and fuse with lysosomes. Following proteolysis of TG, T_3 and T_4 are released into the plasma.

T_4 is the most abundant iodoamine in plasma. Greater than 99% is bound to thyroid-binding proteins such as thyroxine-binding globulin, transthyretin, albumin and apolipoproteins and is unavailable for uptake by target cells. T_3 is

also bound to these proteins but with less affinity. Thus, a higher percentage of T_3 (0.3% vs. 0.03% of T_4) is free in the plasma, suggesting that T_3 is of greater physiological importance. This concept is further supported by the fact that one-third to one-half of T_4 produced by the thyroid is converted intracellularly to T_3 in extrathyroidal tissues. In addition, the calorigenic potential and relative nuclear binding of T_3 is approximately four-fold and ten-fold higher than T_4, respectively. These data suggest T_4 functions largely as a prohormone for the active, intracellular thyroid hormone, T_3[141,142]. It is generally accepted that the majority of the effects of T_3 are mediated transcriptionally *via* thyroid hormone receptors, transcription factors in the nuclear hormone receptor superfamily.

1.5.2 Metabolic functions – Much of what we know about thyroid hormone action in humans has been deduced from the symptoms of thyroid diseases. For instance, the observations that hyperthyroid patients generally have increased appetite, weight loss and excess perspiration indicate that T_3 is important in energy metabolism. Reduced plasma cholesterol during hyperthyroidism[143] suggests a role in lipid metabolism. Given the central role of bile acids in cholesterol and energy metabolism (*via* the catabolism of cholesterol and the facilitation of fatty acid/energy uptake, respectively), the following sections describe the action of T_3 on these processes.

1.5.2(i) Energy metabolism – Hyperthyroid patients display increased basal metabolic rates and oxygen consumption in most tissues. This is

associated with an increased number and size of mitochondria, although the mechanisms of these changes are not clear. Increased thermogenesis and basal body temperature as well as weight loss (despite increased appetite) are other common features of these patients. These examples of increased energy expenditure are caused by a number of factors. T_3 increases ATP hydrolysis by increasing Na^+,K^+-ATPase and Ca^{2+}-ATPase activity. Reduced ATP levels results in increased ATP synthesis *via* glycolysis and glycogenolysis[144,145]. Heat is produced as the Na^+, K^+, and Ca^{2+} gradients dissipate across their respective membranes. T_3 also induces the expression of uncoupling proteins and heat is produced as proton influx into the mitochondrial matrix is dissociated from ATP synthesis[146-148]. Weight loss during hyperthyroidism results primarily from reduced lean body mass due to a net increase in protein degradation[149]. Although appetite is increased, plasma leptin levels are typically normal[150].

1.5.2(ii) Lipid metabolism – As noted above, T_3 induces thermogenesis in brown adipose tissue. White adipose is also affected by T_3. Thyroid hormone regulates both lipogenesis and lipolysis[151] thereby affecting adipose mass. Thyroid hormone can also induce differentiation of preadipocytes into adipocytes[152]. Thus, T_3 has a number of important effects on fatty acid and triacylglycerol metabolism in adipose. Thyroid hormone also regulates lipogenesis and lipolysis in the liver, and in addition regulates cholesterol metabolism. One of the best characterized changes in cholesterol metabolism is the induction of the low density lipoprotein (LDL) receptor (LDLR)[87,153] which

results in increased LDL clearance from the plasma and reduced plasma cholesterol levels. The levels of high density lipoprotein (HDL)-associated cholesterol are also reduced in hyperthyroid patients[143]. The mechanism of this effect is not clear as hepatic expression of apolipoprotein A-I (apoA-I), the main protein component of HDL, is increased by T_3[154,155].

1.5.3 Thyroid hormone dysfunction and bile acid metabolism – Analysis of bile acid metabolism in hypo- and hyperthyroid patients has revealed that the effects of T_3 are limited primarily to the amount and type of bile acids produced. Miller *et al.* observed that the output of bile acids into the duodenum is reduced in hyperthyroid patients and that therapy to reverse the hyperthyroidism caused an increase in bile acid output[156]. Pauletzki *et al.* found that the synthesis of cholic acid, the primary bile acid produced by the neutral pathway, was reduced by 34% in hyperthyroid patients[157]. Kosuge *et al.* observed a reduction in serum cholic acid and deoxycholic acid (a derivative of cholic acid produced in the gut) while total serum bile acids remained unchanged[158]. In addition, cholic acid synthesis tended to be reduced in primary human hepatocytes from five donor livers[90]. These data suggest that bile acid synthesis in humans is repressed by T_3 and is consistent with the T_3-dependent changes of human cyp7a activity and mRNA abundance described in Section 1.4.3. In contrast, changes in bile acid pool size or acid sterol output is not observed in two reports describing the affect of T_3-status on bile acid metabolism[159,160]. Thus, the true effects of T_3 on bile acid synthesis *in vivo* are poorly understood.

The relative proportions of bile acids produced are also affected by T_3. Numerous investigators have observed that T_3 causes a shift in the bile acid profile from CA-derived bile acids to CDCA-derived bile acids in humans[157-159,161]. These data imply that cyp8b1 activity is reduced during hyperthyroidism and are consistent with the ability of T_3 to repress cyp8b1 mRNA abundance in rodents [see Section 1.4.3(iii)].

1.6 Aims

As described above, fundamental differences exist in the regulation of bile acid synthesis in rodents and humans. In rodents, T_3 increases bile acid synthesis *via* increased *Cyp7a1* gene expression and cyp7a activity. In contrast, bile acid synthesis (an indirect measure of cyp7a activity) and cyp7a activity appear to be reduced in hyperthyroid humans. Further, cyp7a mRNA abundance and promoter activity is reduced by T_3 *in vitro*. To directly examine the effects of T_3 on the regulation of human cyp7a, the following aims are proposed:

i) Determine the molecular basis of T_3-mediated repression of the human *CYP7A1* gene,

ii) Construct and validate a transgenic model of human *CYP7A1* gene expression in mice

iii) Directly examine the effect of T_3 on human cyp7a activity and mRNA abundance *in vivo* using the transgenic mouse model

Chapter 2

MATERIALS AND METHODS

2.1 REAGENTS

Tissue culture reagents, restriction endonucleases, DNA-modifying enzymes and markers for agarose gel electrophoresis/SDS-PAGE were purchased from Life Technologies (Gaithersburg, Maryland). McArdle RH7777 (RH7777) cells were obtained from ATCC (Rockville, Maryland). Rodent diets were purchased from ICN Biomedicals, Inc. (Aurora, OH). All other reagents were of analytical grade.

2.2 CLONING

2.2.1 Promoter-reporter gene chimeras – The parental gene chimera (phcyp7a-CAT) was described previously[55]. It features pCAT-basic (Promega Corp., Madision, WI) backbone and the proximal promoter region of the human *CYP7A1* gene (nt –372 to +61) fused to the chloramphenicol acetyl transferase (CAT) structural gene sequence. Mutant gene chimeras were produced from phcyp7a-CAT in which the 5' half-site of Site II (nt –144 to –139) or the 3' half-site of Site III (nt –238 to –233) was mutated to the sequence 5'–CTCGAG–3' (recognition sequence of *Xho* I). Mutagenic sense and antisense oligonucleotides containing *Xho* I recognition sequences at the 5'-end were used to amplify the entire phcyp7a-CAT plasmid by the polymerase chain reaction (PCR) with the high-fidelity polymerase *Pfu* Turbo (Stratagene, La Jolla, CA) using the manufacturer's instructions[viii]. The linear 4.7 kilobasepair (kb) product was

[viii] PCR-based mutagenesis was performed using the following thermocycling profile: denaturing, 1 min at 99°C; annealing, 2 min at 42°C; extension, 5 min at 72°C. The total number of cycles was 25, and the extension time was increased by 5 min at cycles 6, 11, 16, and 21.

digested with *Xho* I and recircularized with DNA ligase following protein removal with Ultrafree Probind spin column (Millipore, Bedford, MA). Clones obtained after transformation were screened by restriction enzyme analysis. The gene chimera pM1.CAT carries the mutation at Site II and was produced using the following oligonucleotides: 5'-TAC CTG CTC GAG TAG TTC AAG GCC AG, 5'-TAC TCG AGC AGG TAT CAG AAG TGG. The gene chimera pM16.CAT carries the mutation at Site III and was produced using the following oligonucleotides: 5'-CCC TCG AGG AAT GTT AAG TCA AC, 5'-ATT CCT CGA GGG GGA CAA CAG C. Another mutant, pM3.CAT, containing a 5'-AAA substitution of nt –134 to –136, was produced using mutagenic sense (5'-TAG CTG TTG TAA ACA GGT CCG A) and antisense (5'-ATT CGG ACC TGT TTA CAA CAG) oligonucleotides and phcyp7a-CAT as the template DNA. Following PCR, the template plasmid DNA was digested with *Dpn* I. The 4.7 kb PCR product was then phosphorylated with T_4 polynucleotide kinase and ligated following protein removal. The primary structure of all mutant gene chimeras was confirmed by DNA sequencing.

A gene chimera featuring the pCAT-basic backbone and the murine *Cyp7a1* gene promoter (nt -412 to -+77) fused to the CAT structural gene sequence has been described previously[55].

2.2.2 Mammalian expression vectors encoding nuclear receptors – All wild type and mutant nuclear receptors were cloned into the vector pSG5 (Stratagene) which features the early SV40 promoter for expression in cell systems and a T_7 bacteriophage promoter for *in vitro* expression. Bacterial

cultures transformed with pSG5-based plasmids encoding human LXRα, human RXRα, and murine PPARα were available in the laboratory of Dr. L. Agellon. The cDNA encoding rat TRα1 was subcloned from a pre-existing vector into the *Eco* RI site of pSG5 and the orientation of the insert verified by restriction enzyme analysis. The primary structure of the entire TRα1 cDNA was confirmed by DNA sequencing. A mutant pSG5-TRα1 expression vector containing a mutation in codon 213 (leucine to alanine) was produced using mutagenic sense (5'-GAT CAT CCT GGC GAA GGG CTG C) and antisense (5'-GCA GCC CTT CGC CAG GAT GAT C) oligonucleotides as described above for pM3.CAT.

2.3 *CELL CULTURE AND ANIMALS*

2.3.1 Animals, treatments and sample collection – Wild type (C57BL6/J) and transgenic mice were maintained *ad libitum* on a normal rodent chow diet (Purina rodent chow 5001). Age-matched mice (8 to 12 weeks) were switched to a reverse light cycle (6:00 AM off, 6:00 PM on) and acclimated for 1 week prior any experiments. In diet studies, the rodent chow was supplemented (w/w) with 1% cholesterol or 0.5% taurocholic acid. This regimen continued for two weeks.

Changes in thyroid status were induced as previously described[88]. Mice were separated into 3 treatment groups and maintained on a low-iodine diet for the remainder of the experiment (see Figure 5-1). On day 7, 2 treatment groups received oral administration of 0.05% 2-mercapto-1-methylimidazole (methimazole) and 1% potassium perchlorate (Sigma-Aldrich Canada Ltd., Oakville, ON). On day 21, 0.1 ml of carrier (0.9% sodium chloride, 0.005 M

sodium hydroxide) or T_3 (0.05 mg/mL in carrier, Sigma-Aldrich) was administered by intraperitoneal injection at the end of the light cycle. Single daily injections continued for 5 days. The experiment was terminated on day 26 as described above.

Animals were deprived of food approximately 8 hours prior to the completion of all experiments. Mice were then sacrificed between 9:00 AM and 11:00 AM following gaseous anesthesia. Blood was collected *via* heart puncture using EDTA-treated needles and syringes after exposure of the chest cavity. Within 3 hours, plasma was separated from all blood samples by low-speed centrifugation and stored at 4°C. Bile was collected by fine needle (27 gauge) puncture of the gallbladder and stored at -80°C. Internal organs were removed, flash frozen in liquid nitrogen and stored at -80°C.

All procedures were performed in accordance with the guidelines of the University of Alberta Health Sciences Animal Policy and Welfare Committee.

2.3.2 Transfections – RH7777 cells were cultured at 37°C/5% CO_2 in 100 mm culture dishes in complete media (DMEM containing 10% carbon-stripped calf serum, 10% carbon-stripped fetal bovine serum) or, where stated, in complete media diluted 1:1 (v/v) with DMEM. Cells from confluent dishes were plated to 60 mm culture dishes and grown to 50% confluence (18 h). The culture medium was aspirated and 20 μg of DNA was transfected using the calcium phosphate co-precipitation method[162]. The DNA mixture consisted of 5 μg of human or murine cyp7a promoter-reporter gene chimera and 5 μg of a plasmid

encoding β-galactosidase under the control of the cytomegalovirus promoter. The DNA mixture also included 5 μg of plasmid encoding the stated nuclear receptor(s): rat TRα1, human RXRα, murine PPARα, human LXRα. The total mass of DNA was adjusted to 20 μg using sheared salmon sperm DNA.

Transfected cells were incubated overnight in complete media diluted 1:4 (v/v) in DMEM. The cells were washed with phosphate-buffered saline (PBS, 137 mM NaCl, 10.1 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.4) and supplied with complete media containing T_3 (10^{-9} to 10^{-5} M), WY14643 (PPARα agonist; 50 μM; Biomol Research Laboratories, Inc., Plymouth Meeting, PA) or 25-hydroxycholesterol (LXRα ligand; 5 μM; Steraloids, Newport, RI). Control cells received either ethanol (0.33%, v/v; carrier for T_3 and 25-hydroxycholesterol) or dimethyl sulfoxide (0.33%, v/v; carrier for WY14643). Following 18 h of treatment, cells were washed with PBS. Cell lysates were prepared by 3 freeze-thaw cycles and assayed for CAT[163] and β-galactosidase[164] activities. The CAT activity in transfected cell lysates was quantitated by phosphorimaging on a Molecular Imager FX Pro Plus phosphorimager (Bio-Rad Laboratories, Hercules, CA) using the Quantity One software package (Bio-Rad) and normalized to β-galactosidase activity.

2.4 Lipid Analysis

2.4.1 Cholesterol – Biliary and plasma cholesterol was measured with the Infinity Cholesterol Reagent (Sigma Diagnostics, Inc., St. Louis, MO) using manufacturer's protocol. To measure lipoprotein cholesterol profiles, equal

amounts of all plasma samples from a treatment group were mixed. Lipoprotein cholesterol profiles were obtained from these pooled samples by high performance gel filtration chromatography as previously described[165]. Briefly, plasma lipoproteins were separated by size exclusion chromatography on a high performance liquid chromatography system (Beckman Instruments (Canada), Inc., Mississauga, ON) fitted with a Superose 6 gel filtration column (Amersham Pharmacia Biotech, Inc., Baie d'Urfé, Quebec). Lipoprotein fractions were then mixed with the Infinity Cholesterol Reagent using a post-column T-connector and passed through a 12-inch line heater set at 37°C. Reaction products were monitored in real-time at 500 nm using a visible-light detector (Beckman).

2.4.2 Bile acids – Gallbladder bile acid mass was measured with the Bile Acid Reagent (Sigma Diagnostics) using the manufacturer's protocol. Bile was diluted 1:2000 or 1:4000 (v/v) in 0.9% sodium chloride prior to measurement. Bile acid speciation in gallbladder bile was determined using an isocratic HPLC method as previously described[166]. Briefly, bile was separated on a LUNA 5 μ C18 column (Phenomenex, Torrance, CA) and detected with a Model 2000 evaporative light scattering detector (Mandel Scientific Co. Ltd., Guelph, Ontario). The identity of the peaks observed was ascertained by comparing the retention times to a set of purified bile acid standards.

2.4.3 Free T_3 – Free T_3 levels in the plasma were measured with the ELISA-based *ACTIVE* Free T_3 EIA kit (Diagnostic Systems Laboratories,

Webster, TX) using the manufacturer's protocol.

2.5 *Nucleic Acid analysis*

2.5.1 Nucleic acid isolation – Various techniques were used for isolating nucleic acids. The isolation of genomic DNA, cloned DNA, and total RNA are described separately.

Genomic DNA

Murine genomic DNA was prepared from tail biopsies following overnight incubation with Proteinase K (Sigma-Aldrich) at 56°C. The solution of proteolyzed tissue was cooled and the chromosomal DNA precipitated with high salt. The DNA was further purified by phenol:chloroform (1:1 v/v) extraction and acetate/ethanol precipitation[167].

Human genomic DNA was prepared from blood donated by healthy male volunteers. Whole blood was centrifuged at 2200xg and the upper plasma fraction removed. The 'buffy coat' fraction, as well as contaminating red blood cells, were collected with a Pasteur pipette and layered onto a cushion of histopaque (Sigma-Aldrich). After a low-speed spin, the isolated leukocytes were transferred to a fresh tube, washed with PBS and collected by centrifugation (2000xg). The DNA was liberated from the cells during an overnight incubation at 56°C in lysis buffer (1 mM EDTA, 10mM Tris-HCl pH 7.8, 10 mM NaCl, 1% SDS, 100 μg/mL proteinase K) and purified by four extractions with phenol:chloroform (1:1 v/v). The aqueous phase was extracted once with chloroform and adjusted

to 0.3 M sodium acetate. The genomic DNA was precipitated with two volumes of ethanol and recovered from solution with a sterile glass rod. The DNA was rinsed in 70% ethanol, dried at room temperature and resuspended in low TE (10 mM Tris-HCl pH 7.4, 0.1 mM EDTA pH 8.0) with an overnight incubation at 37°C.

CLONED DNA

A bacterial artificial chromosome (BAC) library created with human genomic DNA was commercially screened[ix] by PCR with oligonucleotides specific for the 3'-untranslated region of the human *CYP7A1* gene (sense, 5'-CAT TTT GCC GCC ATT GAA TG; antisense, 5'-GAT ATA TGA TCA CAC CCG AAG). BAC DNA was isolated from transformed bacterial cultures using the Plasmid Maxi Kit (Qiagen Inc., Valencia, CA) as per the manufacturer's protocol. Where stated, purified BAC DNA was further purified by gel filtration on Sepharose CL4B column (Amersham-Pharmacia) equilibrated with injection buffer (10 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 0.1 M NaCl). BAC DNA prepared in this manner is suitable for restriction enzyme analysis and injection into zygotes for transgenesis[168] (see Section 4.2.2).

Plasmids for transfections or *in vitro* expression were isolated from transformed bacterial cultures by ultracentrifugation in a cesium chloride gradient containing ethidium bromide[167]. Isolated plasmids were extracted with water-saturated *n*-butanol to remove the ethidium bromide and recovered by acetate/ethanol precipitation. All plasmids for transfection were also sterilized

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[ix] The screening was performed by Genome Systems Inc. (now known as Inctye Genomics, (Palo Alto, CA) using a PCR protocol I developed (see Section 2.5.3).

through a 0.22 μ filter (Millipore).

TOTAL CELLULAR RNA

Total RNA was isolated from murine tissue as previously described[51] using solutions treated with diethyl pyrocarbonate (DEPC; Sigma-Aldrich) and glassware baked at 200°C. Briefly, frozen tissues were homogenized by high-speed agitation in denaturing solution (2.12 M guanidine thiocyanate, 13.20 mM sodium citrate, 18.00 mM *N*-lauroyl sarcosine, 0.70% (v/v) 2-mercaptoethanol) using the model FP120 Fast Prep cell disruptor (Bio 101/Qbiogene, Carlsbad, CA) and RNAse-free tubes containing ceramic beads. The homogenate was extracted with phenol/chloroform, precipitated with isopropanol, and resuspended in DEPC-treated water.

2.5.2 Agarose gel electrophoresis – Native and denaturing agarose gel electrophoresis was performed to analyze nucleic acids. Native gels (0.8 to 1.2% agarose) were used to analyze DNA. Standard (continuous) electrophoresis in 1X TBE (90 mM Tris-borate, 2 mM EDTA pH 8.0) was used for most experiments, but pulsed-field (discontinuous) electrophoresis in 0.5X TBE (200 volts, 20 h, 14°C, 1 sec to 8 sec ramping) was used where stated. Agarose gel loading buffer[167] was added to all DNA samples to a final concentration of 1X (0.04% bromophenol blue, 0.04% xylene cyanol FF, 2.5% Ficoll 400). Denaturing gels (1.2% agarose, 1X MOPS (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA pH 8.0), 4.6% formaldehyde) in 1X MOPS were used to analyze RNA.

Prior to loading, RNA samples were diluted with the following reagents and heated at 60°C for 10 min (final concentrations are indicated): 1X MOPS; 3.7% formaldehyde; 40% formamide. All nucleic acid gels were stained with ethidium bromide and photographed during UV illumination.

2.5.3 Mouse genotyping – Genomic DNA was prepared from tail-biopsies of all mice of interest. Incorporation of the human *CYP7A1* gene into the genomes of mice was assessed by PCR analysis of genomic DNA using human *CYP7A1* gene-specific primers[169] (sense: 5'-TCT GAA CTT GAT CAC CGT CTC TCT G; antisense: 5'-GTC GAC CAA ATC TAG GCC AAA ATC T). The zygozity of the wild type- and null- alleles of murine *Cyp7a1* was determined using a primer pair specific for exon 3 which is absent in the null-allele (sense: 5'-TCA CAA GGT GCG TCT TAG CC; antisense: 5'-GAT GTA TGC CTT CTG CTA CCG) and a primer pair specific for the neomycin phosphotransferase gene (*neo*^{r}) which is absent in the wild type allele (sense: 5'-CTT GGG TGG AGA GGC TAT TC; antisense: 5'-AGG TGA GAT GAC AGG AGA TC). The murine *Mos* gene[170] served as the internal control target in the PCR analyses of mouse genomic DNA (sense: 5'-GGA ATT CAG ATT TGT GCA TAC ACA GTG ACT; antisense: 5'-GTA AAC ATT TTT CGG GAA TAA AAG TTG AGT). The results of PCR were visualized by agarose gel electrophoresis. All PCR screening was performed with *Taq*[x] (Sigma-Aldrich). This PCR-based screening method was validated by DNA blotting and solution hybridization (see below) with a radiolabeled cDNA

[x] PCR-screening of mice was performed using the following thermocycling profile: denaturing, 1 min at 99°C; annealing, 2 min at an optimized temperature; extension, 5 min at 72°C. The total number of cycles was 29.

encoding rat cyp7a.

2.5.4 Nucleic acid blotting and solution hybridization – Where indicated, DNA was incubated with restriction enzymes overnight at 37°C and analyzed by agarose gel electrophoresis. The DNA in the gel was cross-linked with UV irradiation and denatured by sequential incubation of the gel in Solution 1 (0.25 M HCl), Solution 2 (0.5 M NaOH, 1.0 M NaCl) and Solution 3 (3.0 M NaCl, 0.5 M Tris-HCl pH 7.4). The DNA was transferred to Hybond N+ nylon membranes (Amersham-Pharmacia) by capillary transfer[171] in 10X SSC (1.5 M NaCl, 0.15 M trisodium citrate, 12.5 mM EDTA, pH 7.4). RNA was also blotted to Hybond N+ by capillary transfer. Following denaturing agarose gel electrophoresis, the gel was incubated in DEPC-treated 20X SSC for 45 min. DEPC-treated 20X SSC was also used as the transfer buffer.

Membrane hybridization was performed in rotating glass bottles at 55°C and DNA probes labeled with [α-^{32}P]-dCTP (Amersham-Pharmacia) were prepared by the random priming method[167]. All blots were incubated for at least 8 h in HYB buffer (0.25 M sodium phosphate buffer pH 7.2, 7% SDS, 0.1% sodium pyrophosphate, 2 mM EDTA) and then in HYB buffer containing the radiolabeled probe overnight (the probe was diluted in HYB buffer and heated to 100°C for 5 minutes immediately prior to addition). Non-specific binding of the probe to the nylon was then removed from the hybridized blots by sequential stringency washes: 30 min at room temperature in 2X SSC, 0.1% SDS; 20 min at room temperature in 1X SSC, 0.1% SDS; 20 min at 60°C in 0.5X SSC, 0.1% SDS. The

washed blots were sealed in cling film and the results visualized by autoradiography or phosphorimaging as described in Section 2.3.2.

2.5.5 Reverse Transcriptase PCR (RT-PCR) and determination of mRNA abundance – Complementary DNA was synthesized from 10 μg of total RNA with Superscript II Reverse Transcriptase (Invitrogen Canada, Inc., Burlington, ON) using the manufacturer's protocol. PCR was performed in the presence of 1X SYBR Green I (Sigma-Aldrich) with Red *Taq*[xi] (Sigma-Aldrich) and intron-spanning oligonucleotides (Table 2-1) specific for the human *CYP7A1* gene and the following murine genes: *Cyp7a1*, *apoA-I*, *Cyp27*, ATP binding cassette

Table 2-1. SENSE AND ANTISENSE OLIGONUCLEOTIDES USED RT-PCR ANALYSIS.

Target cDNA	Primer Sequences	Size (basepairs)
human cyp7a	5'- TAG CTC TTT ACC CAC AGT TAA TGC -3' 5'- TGG ACC TGG TGA ATC ATT TCT ACC -3'	472
murine cyp7a	5'- AGG AAA GTA GGT GAA CCT CC -3' 5'- TGC TTC TGT GTC CAA ATG CC -3'	281
murine apoA-I	5'- GAA AGC TGT GGT GCT GGC CG -3' 5'- CCT TGT TCA TCT CCT GTC TCA CCC -3'	351
murine cyp27	5'- TGG TTC CCA CAA ACT CCC GGA TCA T -3' 5'- CCA TAG CCA AAG GGC ACA GAG CCA A -3'	222
murine ABCAI	5'- GAC GAT ATC TCG ATT CAT GG -3' 5'- CAC GTT GTC AAT GTC CAT CC -3'	201
murine LDLR	5'- CCG TCC TGC TGT GGA GGA AC -3' 5'- TAG GTA TAG CCA TCC TGG CT -3'	130
murine HMGR	5'- AAG ATC TTC AAG GAA CGT GC -3' 5'- GTC ACT GAT CAT GTC CAT TC -3'	154
murine cyclophilin	5'- TCC AAA GAC AGC AGA AAA CTT TCG -3' 5'- TCT TCT TGC TGG TCT TGC CAT TCC -3'	356

[xi] PCR-based analysis of mRNA abundance was performed using a convection Lightcycler and the following thermocycling profile: denaturing, 0 sec at 99°C; annealing, 20 sec at an optimized temperature; extension, 20 sec at 72°C.

transporter protein A1 (*ABCA1*), *LDLR*, HMG-CoA reductase (*HMGR*) and cyclophilin. The annealing temperature of each oligonucleotide pair was optimized using a T-Gradient thermal cycler (Biometra, Göttingen, Germany) to ensure the synthesis of only one DNA product.

The results of PCR using some of the oligonucleotides pairs are shown in Figure 2-1A. In general, the size of each amplicon is different to unambiguously identify the PCR product. Amplicon production was monitored to determine the range of cycles in which amplification remained linear. This is illustrated in Figure 2-1B for the ABCA1 amplicon. SYBR Green I is a dye which fluoresces when bound to double-stranded DNA. Amplicon production was monitored after a pre-determined number of PCR cycles by green fluorescence using a LightCycler

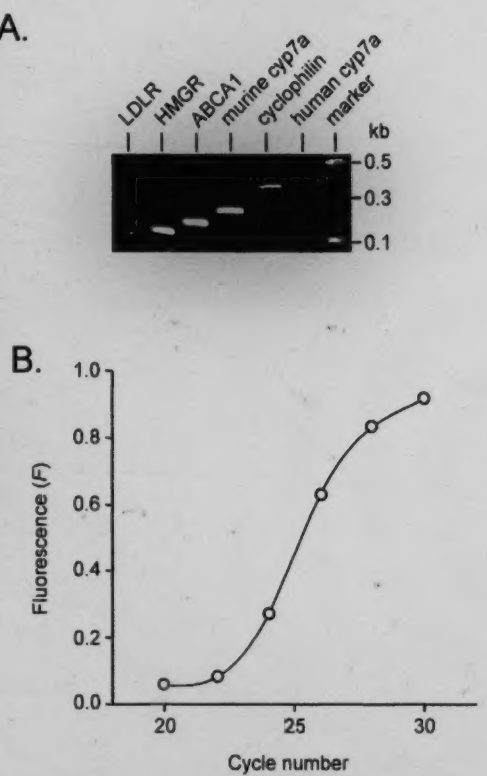


Figure 2-1. RT-PCR ANALYSIS OF VARIOUS HEPATIC mRNA SPECIES. ***A,*** Oligonucleotides for the indicated genes were used to amplify specific cDNA fragments by PCR. The amplicons were analyzed by agarose gel electrophoresis. ***B,*** Amplicon production using oligonucleotides specific for the ABCAI cDNA was measured by green fluorescence after the indicated number of PCR cycles.

(Roche Diagnostics Canada, Laval, Quebec). Fluorescence due to amplicon production was differentiated from non-specific fluorescence by melting curve analysis.

As shown in Figure 2-2A, the total fluorescence at 68°C following PCR was comparable between reactions with and without the addition of cDNA template. However, as the temperature is increased, practically all of the fluorescence is lost in the sample which did not receive any template cDNA. A second drop in fluorescence beginning at approximately 84°C is visible in the sample which received cDNA template. This effect is more pronounced when the inverse derivative of the fluorescence is calculated and plotted as a function of

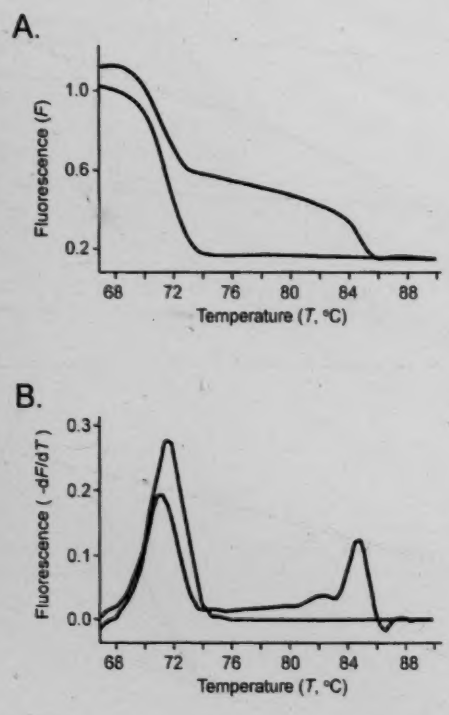


Figure 2-2. ANALYSIS OF MRNA ABUNDANCE BY GREEN FLUORESCENCE. ***A,*** Raw fluorescence (*F*) obtained during melting curve analysis of the ABCA1 amplicon. ***B,*** The change in fluorescence during melting curve analysis (d*F*/d*T*) was calculated from *A*. The PCR reactions analyzed were performed using oligonucleotides specific for the ABCA1 cDNA in the presence (red line) or absence (blue line) of reverse transcribed total hepatic RNA. Fluorescence due to the ABCA1 amplicon is detected only in the presence of reverse transcribed total hepatic RNA at approximately 84.5°C. Non-specific fluorescence is detected in both samples at approximately 72°C.

temperature (Figure 2-2B). Amplicon mass is thus quantitated from the inverse derivative of the fluorescence by integrating the area under the peak corresponding to the expected amplicon using the LightCycler software package (Roche). All reactions were then confirmed visually by agarose gel electrophoresis. Cyclophilin mRNA abundance varied less than 10% among all samples and was used to normalize the mRNA levels of the other genes.

2.6 *PROTEIN ANALYSIS,* IN VITRO *SYNTHESIS, AND ENZYME ASSAYS*

2.6.1 Protein immunoblotting – Proteins were separated by SDS-PAGE[172], transferred to a PVDF membrane (Millipore Corporation, Bedford, MA) using the manufacturer's protocol and detected using rabbit polyclonal antibodies or rabbit antiserum. Nuclear receptors were detected using antibodies raised against the TRs (sc-772, Santa Cruz Biotechnology Inc., Santa Cruz, CA; detects TRα1 and TRβ1 isoforms) or RXRs (sc-774, Santa Cruz Biotechnology Inc.; detects RXRα and RXRβ isoforms). Cyp7a was detected using antiserum raised against the last 5 carboxyl terminal amino acid residues of the human cyp7a enzyme[169]. The primary antibody-antigen complexes were visualized with the ECL-based detection system (Amersham Pharmacia Biotech, Baie d'Urfé, Quebec) using peroxidase-conjugated anti-rabbit IgG as the secondary antibody.

Three protein sources were used for protein immunoblotting in this research: i) cell lysates from RH7777 cells, ii) liver homogenates from mice and iii) recombinant nuclear receptors expressed *in vitro*. Recombinant TRα and RXRα were prepared using the TNT Coupled Reticulocyte Lysate System

(Promega Corp., Madison, WI) programmed with 0.5 μg of plasmid encoding either rat TRα1[173] or human RXRα[174]. The production of these recombinant proteins in this *in vitro* system was confirmed by performing parallel reactions in which the methionine was replaced with *L*-[^{35}S]-methionine [Amersham-Pharmacia]. The labeled proteins were separated by SDS-PAGE and detected by autoradiography. As shown in Figure 2-3, the plasmids encoding both TRα and RXRα produce proteins of the expected size.

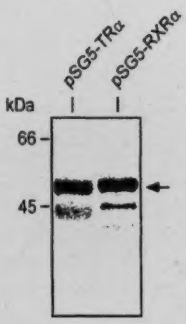


Figure 2-3. EXPRESSION OF NUCLEAR RECEPTORS *IN VITRO*. TRα and RXRα were expressed *in vitro* in the presence of ^{35}S-methionine using the indicated plasmids. The labeled products were analyzed by SDS-PAGE and visualized by autoradiography. The position of known standards is shown at left and the arrow indicates the expected position of the receptors.

2.6.2 Bacterial expression of recombinant receptors - Plasmids encoding nuclear receptors were grown in *E. coli* strain BL21 (DE3) and the recombinant receptor partially purified as described earlier[175]. Briefly, cultures were induced at an OD_{600} of approximately 1.0 with isopropylthiogalactoside for 30 min. Rifampicin was added and the culture incubated for an additional 1 h. The cultures were rapidly chilled, collected by centrifugation and lysed by freeze-thaw in the presence of 0.05% Triton X-100. Cell debris and nucleic acid were removed by centrifugation and precipitation. A 20% to 30% ammonium sulfate fraction was then prepared from the crude protein solution and the expressed

receptors isolated by affinity chromatography on a heparin-agarose column (Sigma-Aldrich). The receptors were eluted from heparin-agarose with GTETD375 (15% glycerol, 25 mM Tris-HCl pH 7.8, 0.5 mM EDTA, 0.05% glycerol, 1 mM dithiothreitol, 375 mM potassium chloride) and concentrated using Ultrafree-15 centrifugal filter devices (Millipore).

2.6.3 Cyp7a activity assay – Microsomes were prepared from frozen liver as described previously[15] following homogenization with a Polytron PT 10-35 (Kinematica AG, Switzerland). Cyp7a specific activity was measured by the conversion of [4-^{14}C]-cholesterol (Amersham Pharmacia) into [4-^{14}C]-7α-hydroxycholesterol as previously described[15]. Sterols were separated by thin layer chromatography and quantitated by phosphorimaging as described in Section 2.3.2. Activity is expressed as pmol of 7α-hydroxycholesterol produced per minute per mg of microsomal protein.

2.7 *Characterization of protein-DNA interactions*

2.7.1 DNAse I footprinting – phcyp7a-CAT (10 μg) was digested with either *Hind* III or *Sal* I in a total reaction volume of 200 μl. The linear DNA was mixed with 6 units of the Klenow fragment of DNA polymerase I, 50 μCi of [α-^{32}P]dATP, 50 μCi of [α-^{32}P]dTTP (3000 Ci/mmol each) and dCTP/dGTP, each to a final concentration of 181 μM, in a total volume of 220 μl. After labeling, the DNA polymerase was heat-inactivated and removed by centrifugation of the reaction mixture through a Ultrafree Probind spin column (Millipore). The labeled

DNA were digested with 100 units of either *Sal* I (after *Hind* III) or *Hind* III (after *Sal* I) to produce fragments with only one labeled strand. The labeled promoter fragments were separated from the plasmid vector by agarose gel electrophoresis and purified by elution from crushed agarose gel plugs[172]. The radiospecific activity of the labeled probe was determined by liquid scintillation spectrometry.

TRα binding sites in the human *CYP7A1* gene promoter were mapped by DNAse I footprinting[176]. Briefly, bacterially expressed TRα was incubated with the radiolabeled promoter fragment in 1X binding buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 10 mM EDTA). The DNA was then partially degraded with DNAse I and analyzed by gel electrophoresis through a 6% acrylamide gel. The labeled DNA fragments were visualized by autoradiography and the locations of the protected regions were deduced using a ladder generated by purine-specific chemical cleavage[172] of the radiolabeled probe.

2.7.2 Electrophoretic mobility shift assays (EMSAs) – Complementary synthetic oligonucleotides were annealed in 1X annealing buffer (100 mM NaCl, 10 mM Tris-HCl pH 7.5, 10 mM EDTA) at a final concentration of 10 pmol/μl. Double-stranded oligonucleotides (20 pmol) or restriction DNA fragments were end-filled in 1X Klenow buffer (10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 7.5 mM dithiothreitol) with 2 U of the Klenow fragment of DNA polymerase I, 667 μM each of dCTP and dGTP, and 50 μCi each of [α-^{32}P]dATP (3000 Ci/mmol) and [α-^{32}P] dTTP (3000 Ci/mmol), in a final reaction volume of 30 μl. After labeling,

the free nucleotides were removed from the reaction mixture by spun-column chromatography[167] through a 2 ml bed of Sephadex G-25. The radiospecific activity of the labeled probe was determined by liquid scintillation spectrometry.

The labeled oligonucleotide was incubated with bacterially expressed, recombinant nuclear receptors and 1 μg of poly(dI-dC)•poly(dI-dC) (Roche Molecular Biochemicals, Laval, Quebec) in 1X binding buffer at room temperature for 20 min. Where indicated, 1 μl of an antibody specific for TRs (above) or an excess of unlabelled competitor DNA was added to the reaction and incubated for an additional 30 min. Protein-DNA complexes were separated from free probe by non-denaturing electrophoresis on a 5% polyacrylamide gel and visualized by autoradiography.

Chapter 3

A DISTINCT THYROID HORMONE RESPONSE ELEMENT MEDIATES REPRESSION OF THE HUMAN *CYP7A1* GENE PROMOTER IN HEPATOMA CELLS*

* Parts of this chapter have been published: Drover VA, Wong NC and Agellon LB (2002) *Mol Endocrinol* **16**:14-23.

3.1 INTRODUCTION

T_3 plays an important role in the regulation of cyp7a. In rodents, cyp7a activity, mRNA abundance and *Cyp7a1* gene expression are stimulated by T_3[79-88]. Surprisingly, analysis of the rat *Cyp7a1* gene promoter in HepG2 cells failed to show a response to T_3 even in the presence of TRα[66]. In contrast, T_3 represses the human *CYP7A1* proximal gene promoter in HepG2 cells[128] and has a tendency to decrease bile acid synthesis and *cyp7a* mRNA abundance in human hepatocytes[90]. Indirect analysis of cyp7a activity *in vivo* has also revealed that 7α-hydroxy-4-cholesten-3-one (see Figure 1-4) in untreated hyperthyroid patients was 27% lower than in untreated hypothyroid patients[130]. Further, CA synthesis, CA pool size and the output of bile acids into the duodenum are reduced during hyperthyroidism and increased by treatments which normalize plasma T_3 levels[156,157]. Thus, T_3 appears to repress cyp7a. In this chapter, the molecular basis of T_3-mediated repression of the human *CYP7A1* gene promoter was examined. The data show that TRα can bind to two sites in the human *CYP7A1* gene promoter *in vitro*. Functional characterization of these binding sites in hepatoma cells revealed that only one site is capable of mediating the repression of the human *CYP7A1* gene promoter by T_3/TRα.

3.2 RESULTS

3.2.1 T_3 represses the human CYP7A1 promoter – To determine the effect of T_3 on the activity of the human *CYP7A1* gene promoter, a gene chimera consisting of the proximal human *CYP7A1* gene fused to the CAT reporter

(phcyp7a-CAT) was transfected into RH7777 cells in the presence and absence of T_3. CAT activity was used as an indicator of promoter activity. Physiological and supraphysiological concentrations of T_3 as high as 10 μM had no effect on CAT activity in RH7777 cells (Figure 3-1A). As T_3 function is mediated by TRs, we surmised that RH7777 cells do not have sufficient amounts of this receptor to allow the regulation of T_3 responsive genes. Accordingly, immunoblot analysis of whole cell protein extracts from RH7777 cells did not reveal the presence of TRs while RXRs were readily detectable (Figure 3-1B). Thus, the lack of change in promoter activity in the presence of T_3 may be attributable to the absence of TRs in RH7777 cells.

When RH7777 cells were cotransfected with a plasmid encoding TRα, T_3 decreased promoter activity in a concentration-dependent manner (Figure 3-1C). T_3 concentrations as low as 100 nM were sufficient to reduce promoter activity by 50%. TRs are transcriptionally active as monomers, homodimers or as heterodimers with RXRs[177-179]. Cells cotransfected with both TRα and RXRα exhibited similar magnitudes of T_3-dependent repression of promoter activity (Figure 3-1D) as observed in cells transfected with TRα only. Thus, repression of the human *CYP7A1* gene promoter by T_3 is dependent upon the presence of TRα.

3.2.2 The human CYP7A1 gene contains two elements that bind TRα – The majority of the effects of T_3 are mediated *via* the binding of TRs to specific DNA sequences in the promoter of target genes. To determine if TRα bound the

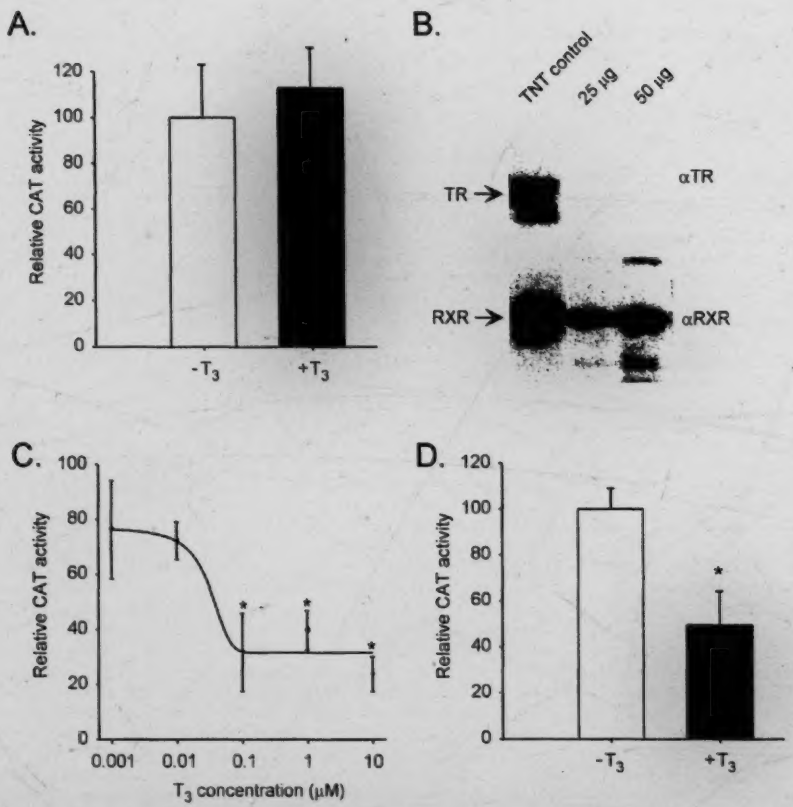


Figure 3-1. ACTIVITY OF THE HUMAN *CYP7A1* GENE PROMOTER IN RH7777 CELLS. ***A,*** phcyp7a-CAT was transfected into RH7777 cells and treated with ethanol (carrier, open bars) or 10 µM T_3 (filled bars) for 18 hours. Following treatment, the cells were harvested and assayed for CAT activity. ***B,*** Whole-cell protein extracts from mock transfected RH7777 cells were prepared and assessed for the presence of TRs and RXRs by immunoblot analysis. Recombinant receptors produced in a cell-free transcription/translation system were used as positive controls (TNT control). The mass of cell extract used in the experiment is shown above the lanes. ***C,*** phcyp7a-CAT was co-transfected into RH7777 cells with an expression vector encoding TRα and treated with the indicated concentrations of T_3 for 18 hours. Following treatment, the cells were harvested and assayed for CAT activity. ***D,*** phcyp7a-CAT was co-transfected into RH7777 cells with expression vectors encoding TRα and RXRα and treated with ethanol (carrier, open bars) or 100 nM T_3 (filled bars) for 18 hours. Following treatment, the cells were harvested and assayed for CAT activity. The activity of the promoter in the absence of T_3 was taken as 100%.The data shown is the mean ± SEM relative CAT activity of triplicate assays from two experiments. * Differences were evaluated using a two-sample *t*-test and were considered significant when $P<0.05$.

the human *CYP7A1* gene promoter, DNA fragments containing Site I, Site II and Site III were prepared using restriction endonucleases (Figure 3-2A). These promoter fragments were labeled and TRα binding was determined by EMSA. As shown in Figure 3-2B, TRα bound the promoter fragment produced with *Ssp* I and *Dde* I and containing Site III (Ss-D, lane 4). The major TRα:Ss-D complex had a similar mobility to the Site A sequence from the apoA-I promoter which is

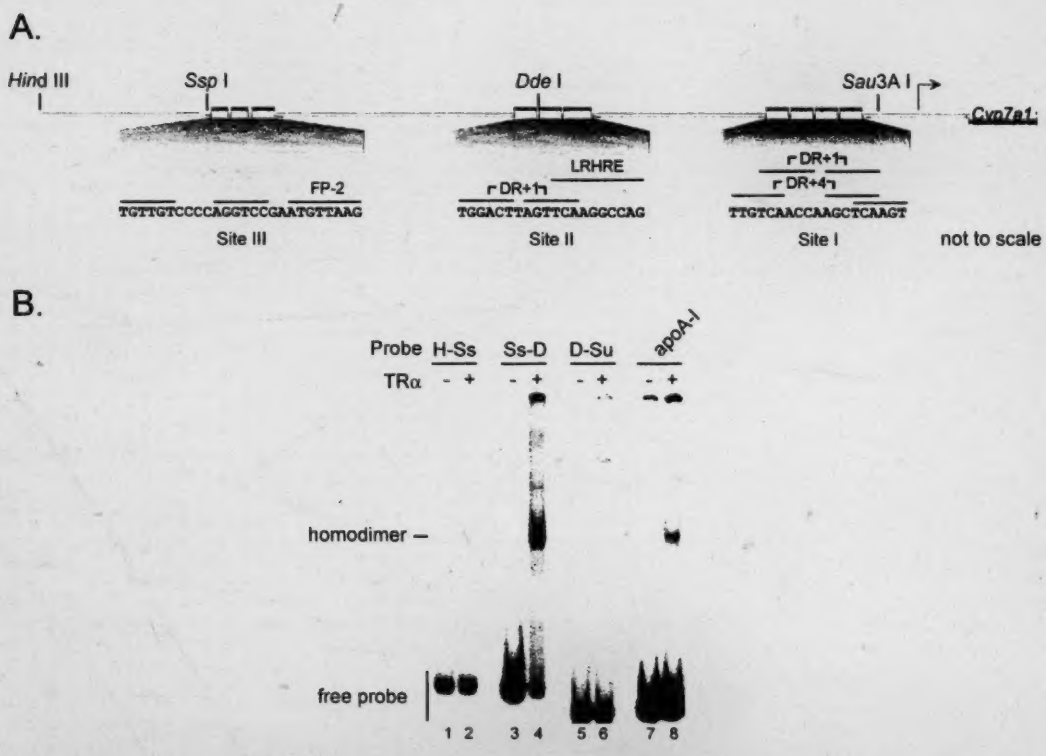


Figure 3-2. TRα BINDS THE HUMAN *CYP7A1* GENE PROMOTER. ***A,*** The human *CYP7A1* gene promoter is illustrated and restriction endonuclease cleavage sites used to produce promoter fragments are indicated. ***B,*** Human *CYP7A1* gene promoter fragments (produced using the indicated restriction endonucleases; H, *Hind* III; Ss, *Ssp* I; D, *Dde* I; Su, *Sau*3A I) were labeled with ^{32}P-deoxynucleotide triphosphates and incubated with or without recombinant TRα. Protein-DNA complexes were separated from free probe by non-denaturing electrophoresis on a 5% polyacrylamide gel. The migration of the free probe and shifted complexes are indicated at the left. Site A from the apoA-I promoter (lanes 7 and 8) is known to bind TR homodimers and was used as a control.

known to bind two molecules of TRα (lane 8)[180]. TRα binding was not detected with the promoter fragment produced with *Hin*d III - *Ssp* I (H-Ss, lane 2) but faint binding was observed with the *Dde* I – *Sau*3A I fragment (D-Su, lane 6). However, these protein:DNA complexes displayed a different mobility than the apoA-I promoter fragment and the Ss-D fragment.

Sequences bound by TRα in the intact human *CYP7A1* gene promoter were mapped using DNAse I protection experiments. Two protected regions were identified. The first region spans nt -119 to -149 (Figure 3-3A) and corresponds to Site II. The second protected region spans nt -227 to -247 (Figure 3-3B) and corresponds to Site III. As TRα can heterodimerize with RXRα,

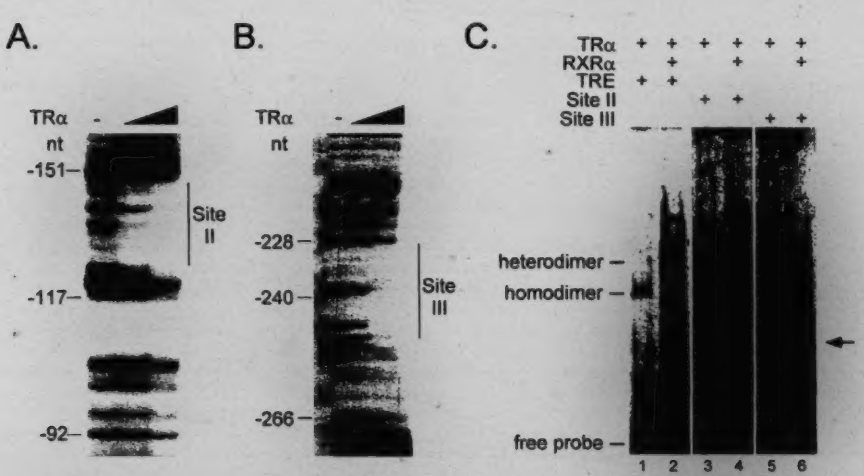


Figure 3-3. MAPPING TRα BINDING SITES IN THE HUMAN *CYP7A1* GENE PROMOTER. ***A*** and ***B,*** Human *CYP7A1* gene promoter fragments labeled in one strand at either the 3'– or 5'–end (panels *A* and *B*, respectively) were incubated with recombinant TRα and partially degraded with DNAse I. The products were analyzed by denaturing electrophoresis on a 6% polyacrylamide gel. Protected regions (Site II and Site III) are indicated by the vertical lines at the right. The corresponding positions in the human *CYP7A1* gene promoter are indicated in nt at the left. ***C,*** Labeled double-stranded oligonucleotides corresponding to TRE, Site II or Site III (see Table 3-1) were incubated with recombinant TRα and RXRα. Protein-DNA complexes were separated from free probe by non-denaturing electrophoresis on a 5% polyacrylamide gel. The migration of the free probe and shifted complexes is indicated at the left.

EMSAs were performed in the presence of both recombinant receptors to determine whether TRα can bind Site II and Site III as a TRα:RXRα heterodimer. TR homodimers can be distinguished from TR/RXR heterodimers by a difference in electrophoretic mobility[181]. When both TR and RXR were incubated with an idealized thyroid hormone response element (TRE; a direct repeat separated by 4 nt), the shifted complex migrated more slowly than when the receptors were incubated with either Site II or Site III (Figure 3-3C, lanes 2, 4, and 6, respectively). These results indicate that Site II and Site III bind only TRα even in the presence of RXRα. The presence of a faster migrating complex in lane 6 (arrow) further suggests that TR may bind as a monomer to Site III (see Fig. 3-5).

3.2.3 Specificity of TRα binding to Site II and Site III – EMSAs were performed to characterize the receptor binding to Site II and Site III. Oligonucleotides containing the putative nuclear hormone receptor binding sites (related to the hexanucleotide sequence 5′–AGGTCA–3′) within Site II and Site III were synthesized and are shown in Table 3-1. Radiolabeled oligonucleotides containing the Site II sequence bound recombinant TRα (Figure 3-4A, lane 2). The TRE competed for TRα binding (lanes 3-6), while a 100-fold excess of a different sequence (the glucocorticoid response element, GRE) had no effect on TRα binding (lane 10). An antibody recognizing TRs was used to further illustrate the specific interaction of Site II with TRα (lane 13). Similarly, Site III bound TRα (Figure 3-4B, lane 2) and was competed by the TRE but not by the GRE (lanes 3-6 and 7-10, respectively). These results demonstrate that TRα can interact

Table 3-1. OLIGONUCLEOTIDES USED FOR EMSAs.

Name	Sequence	Position (nt)
Site II	tacctg**TGGACT**t**AGTTCA**aggccagtt	-150 to -123
Site II m1	......ctcgag.....	
Site II m2	.............ctcgag.....	
Site III	tagc**TGTTGT**cccc**AGGTCC**gaat	-252 to -229
Site III m1	....ctcgag.....	
Site III m2	.............ctcgag....	
Site III m3	..........aaa.....	
Site III m4	....aaa.....	
TRE	gatcctc**AGGTCA**cagg**AGGTCA**gag	
GRE	tcgactgtacagga**TGTTCT**agctact	
F2-TRE	ttat**TGACCC**cagctg**AGGTCA**agttacg	

The sense-strand sequence of oligonucleotides used in EMSAs is shown in the 5' to 3' orientation. The putative nuclear receptor binding sites (related to the sequence 5'–AGGTCA–3') are indicated in bold, uppercase type-face. The position of the wild type oligonucleotides in the human CYP7A1 *gene promoter is indicated with respect to the transcription initiation site. Conserved residues in mutant oligonucleotides are indicated by (.).*

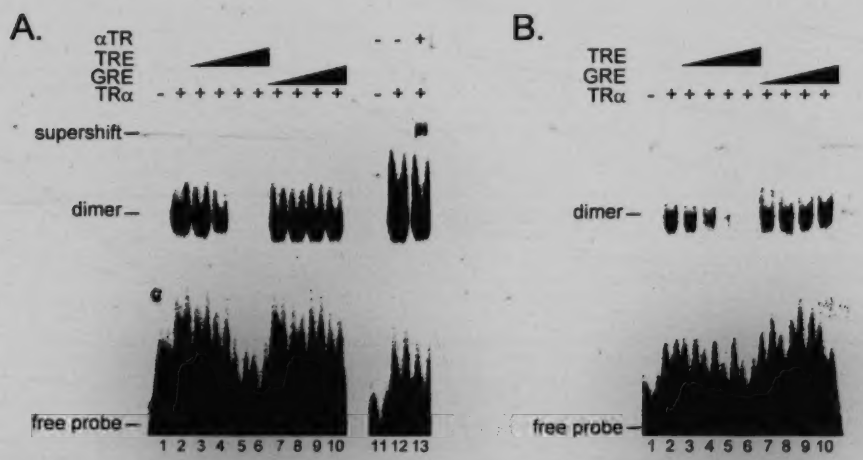


Figure 3-4. BINDING OF RECOMBINANT TRα TO SITE II AND SITE III. Recombinant TRα was incubated with the labeled, double-stranded oligonucleotides (see Table 3-1) corresponding to Site II or Site III (panels ***A*** and ***B***, respectively). Unlabeled specific and non-specific competitors (TRE and GRE, respectively) were added (where indicated) in 0.1, 1, 10, and 100 fold molar excess of the labeled oligonucleotide. An antibody specific for TRs was added where indicated. Protein-DNA complexes were separated from free probe by non-denaturing electrophoresis on a 5% polyacrylamide gel. The migration of TRα homodimers, the free probe and supershifted complexes of TRα homodimers and anti-TR antibodies are indicated at the left.

specifically with two regions in the human *CYP7A1* proximal promoter and confirm the DNAse I footprinting results above.

3.2.4 Putative nuclear receptor binding sites are required for TRα binding to Site II and Site III – The Site II sequence was previously described and features a DR+1 and the LRHRE (see Figure 1-10). LRH-binding to the cyp7a promoter is conserved between species and is required for the liver-specific expression of the *CYP7A1* gene as well as bile acid-mediated repression of gene expression *via* FXR and SHP. Therefore, the LRHRE is most likely occupied and unavailable for TRα binding. The DR+1 was thus considered the most likely target for TRα binding at Site II.

To determine the nature of the interaction between TRα and Site II, mutant oligonucleotides containing substitutions of either of the putative nuclear hormone receptor binding sites within the DR+1 were synthesized (Table 3-1). Mutagenesis of the 5'–hexanucleotide sequence (Site II m1) abrogated TRα binding (Figure 3-5A, lane 4). Only residual binding of TRα was detected when the 3'–hexanucleotide sequence was altered (Site II m2; lane 6). The requirement of both hexanucleotide sequences for efficient receptor binding suggests Site II is bound by two molecules of TRα *in vitro*. In addition, the mobility of a previously characterized TRα dimer (Site A of the ApoA-I promoter[180]; lane 8) matches the mobility of TRα bound to Site II, consistent with the presence of two molecules of TRα at this site.

Analysis of the Site III sequence initially revealed two putative nuclear hormone receptor binding sites separated by 4 nt (Table 3-1). Mutation of the 5'–

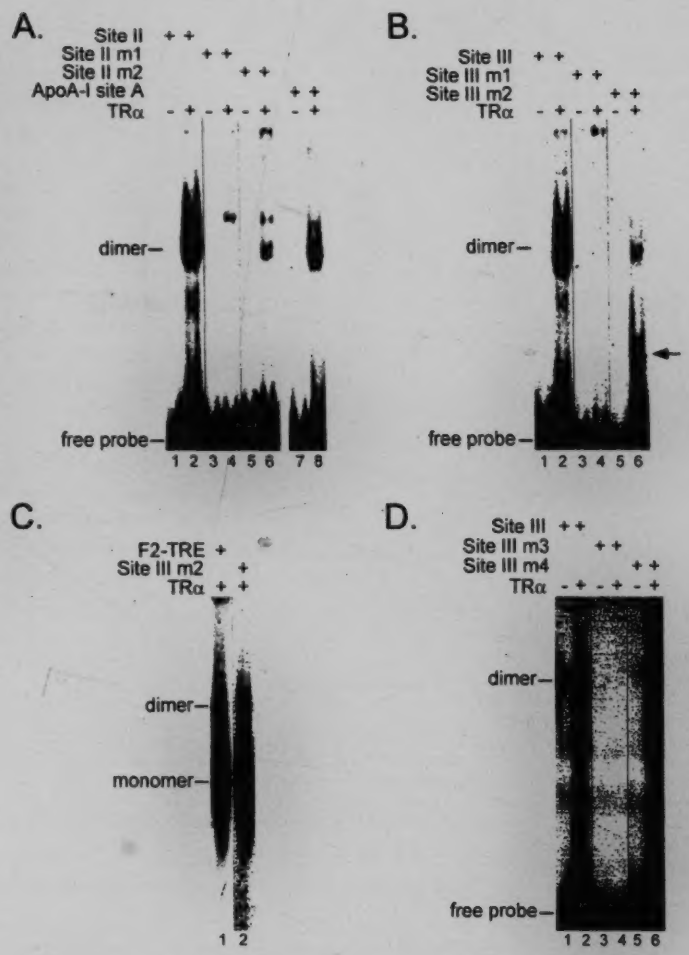


Figure 3-5. SEQUENCE DEPENDENCE OF TRα BINDING TO SITE II AND SITE III. Wild type and mutant labeled, double-stranded oligonucleotides (see Table 3-1) corresponding to Site II (panel ***A***) or Site III (panels ***B***, ***C*** and ***D***) were incubated with or without recombinant TRα. Protein-DNA complexes were separated from free probe by non-denaturing electrophoresis on a 5% polyacrylamide gel. The migration of TRα monomers, dimers and the free probe are indicated at the left. Site A from the apoA-I promoter (panel *A*) and the F2-TRE (panel *C*) were used as markers for TR dimers and monomers (in the presence of 10^{-7}M T_3), respectively.

hexanucleotide sequence (Site III m1) abrogated binding of TRα to Site III entirely (Figure 3-5B, lane 4). Mutation of the 3'–hexanucleotide sequence (Site III m2) resulted in reduced receptor binding (lane 6) and the appearance of a second complex with faster mobility (indicated with an arrow). The mobility of this second complex was the same as that of the F2-TRE from the chicken lysozyme gene (Figure 3-5C) which is known to bind TR as a monomer[182].

Further analysis of Site III revealed the existence of a third putative nuclear hormone receptor binding site (5'–TGTCCC–3') which overlaps the 5'–

hexanucleotide sequence and is separated from the 3'–hexanucleotide sequence by 1 nt. As the mutation in the Site III m1 oligonucleotide also disrupted the overlapping-hexanucleotide sequence, we synthesized oligonucleotides containing more subtle mutations to delineate which sequence was required for TR binding. Substitutions which preserved the 5'–hexanucleotide sequence (Site III m3) abrogated receptor binding (Figure 3-5D, lane 4). Oligonucleotides containing substitutions which preserved the overlapping-hexanucleotide sequence (Site III m4) displayed an electrophoretic mobility comparable to wild type Site III (lane 6). These data show that the overlapping- and 3'–hexanucleotide sequences of Site III (apparently arranged as an everted repeat spaced by one nt) direct the binding of two TRα molecules *in vitro*.

3.2.5 One half-site in Site III independently mediates T_3-dependent repression of the human CYP7A1 gene promoter – Since TRα is able to bind Site II and Site III *in vitro*, gene chimeras containing mutations at these sites were characterized to define their functional significance. A gene chimera containing a substitution at the 5'–hexanucleotide sequence of Site II (pM1.CAT) was created. Like the wild type chimera (Figure 3-6A, phcyp7a-CAT), promoter activity was repressed by T_3 in the presence of TRα (Figure 3-6B). This finding indicates that Site III, the only remaining TRα binding site in pM1.CAT, is sufficient to mediate T_3-dependent repression of promoter activity. A second mutant gene chimera (pM16.CAT) was created in which the 3'–hexanucleotide sequence of Site III was

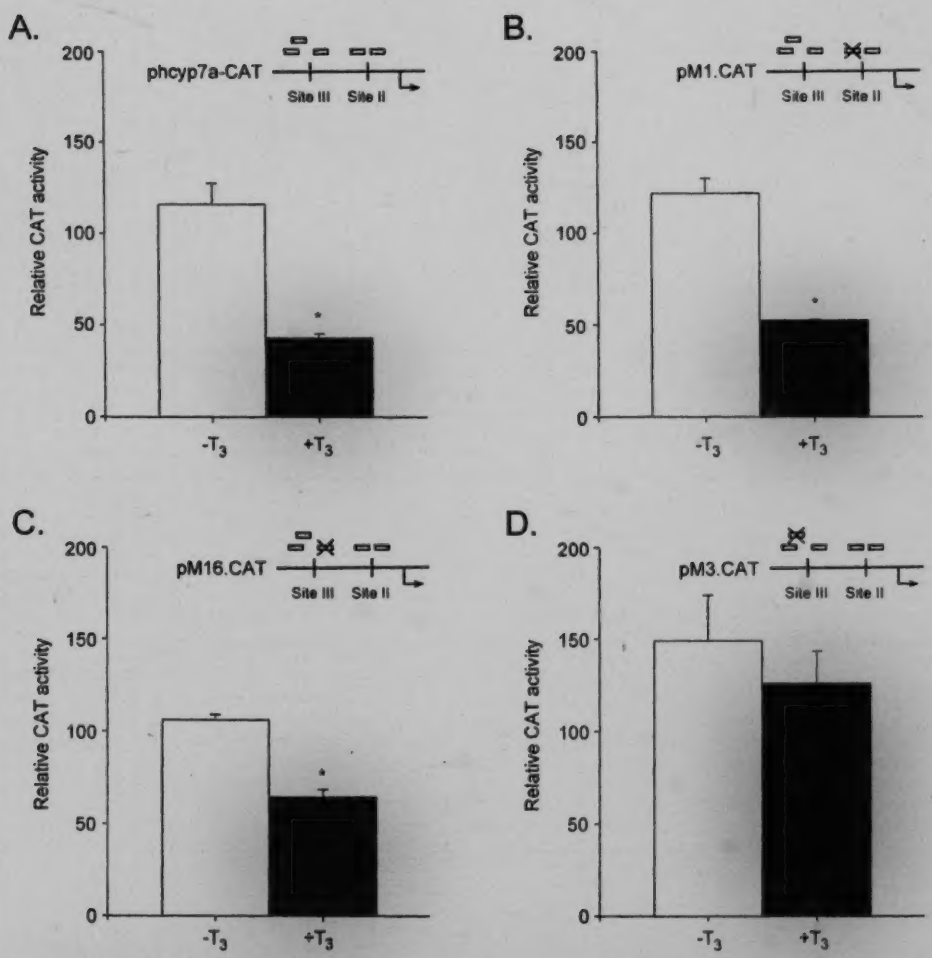


Figure 3-6. FUNCTIONAL ANALYSIS OF GENE CHIMERAS CONTAINING MUTANT SITE II AND SITE III SEQUENCES. RH7777 cells were co-transfected with parental (phcyp7a-CAT; panel ***A***) or mutant (pM1.CAT, pM16.CAT, pM3.CAT; panels ***B***, ***C*** and ***D***, respectively) gene chimeras and an expression vector encoding TRα. Transfected cells were then grown for 18 hours in the presence of ethanol (carrier, open bars) or 100 nM T_3 (filled bars) and assayed for CAT activity. The activities of the promoters in the absence of both T_3 and TR were taken as 100%. The data shown are the mean ± SEM relative CAT activity of triplicate assays from two experiments. The specific activities (pmol · min^{-1} · mg^{-1} total cell protein) are: phcyp7-CAT, 1.68; pM1.CAT, 1.41; pM16.CAT, 1.06; pM3.CAT, 0.6). * Differences were evaluated using a two-sample *t*-test and were considered significant when $P<0.05$. The locations of Site II and Site III are shown schematically above each panel. Open boxes indicate the location of TR-binding half-sites at Site II and Site III. The boxes designated with an 'X' represent mutagenized half-sites.

altered. Surprisingly, the addition of T_3 still resulted in repression of promoter activity (Figure 3-6C).

We hypothesized that the overlapping-hexanucleotide sequence of Site III, which was still intact in both pM1.CAT and pM16.CAT gene chimeras, might be sufficient to mediate the T_3 response. To test this hypothesis, we created another gene chimera (pM3.CAT) in which only the overlapping-hexanucleotide sequence of Site III was altered. This substitution rendered the promoter unresponsive to T_3 (Figure 3-6D). Taken together, the results show that a single hexanucleotide sequence in Site III is necessary and sufficient for the T_3/TRα-mediated repression of the human *CYP7A1* gene promoter.

3.2.6 T_3-dependent repression of the human CYP7A1 gene promoter is altered by mutations in TRα which affect coactivator/corepressor interactions – Genes that are transactivated by ligand-bound nuclear receptors are often repressed by the ligand-free receptor. This is due to the association of the nuclear receptor with transcriptional coregulators which reduce transcription (corepressors) in the absence of ligand (reviewed for TRs in[183]). Ligand binding causes a conformational shift in the receptor which results in the dissociation of the corepressor and recruitment of a transcriptional coactivator. Similarly, transrepression by ligand-bound nuclear receptors is also thought to involve a 'switch' in transcriptional coregulators. Thus, ligand-free TRα should increase cyp7a promoter activity in RH7777 cells. As shown in Figure 3-7, ligand-free receptor significantly increased promoter activity by 52%.

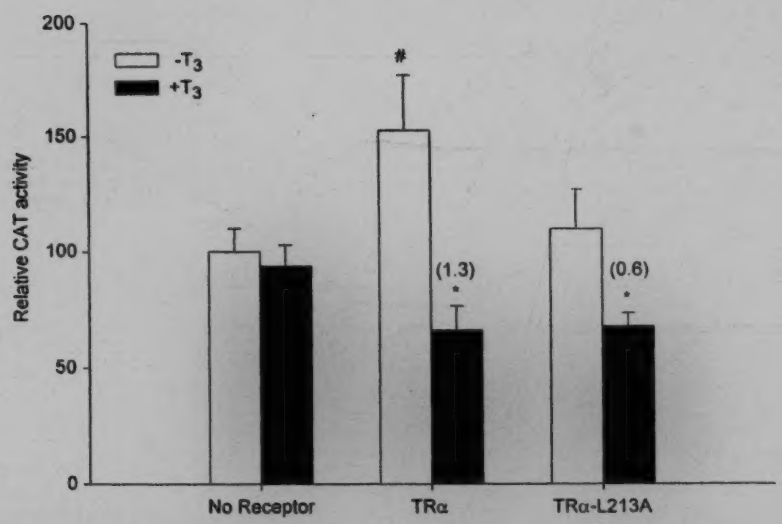


Figure 3-7. TRα-L213A DISPLAYS DEFECTIVE T_3-DEPENDENT REPRESSION OF *CYP7A1* PROMOTER ACTIVITY. RH7777 cells were co-transfected with phcyp7a-CAT and wild type TRα or mutant TRα-L213A. Transfected cells were then grown for 18 hours in the presence of ethanol (carrier, open bars) or 100 nM T_3 (filled bars) and assayed for CAT activity. The activities of the promoters in the absence of both T_3 and TR were taken as 100%. The data shown are the mean ± SEM relative CAT activity of triplicate assays from two experiments. Fold-repression induced by T_3 in the presence of receptor is indicated in parentheses. Differences were evaluated using a two-sample *t*-test and were considered significant when $P<0.05$. #, significantly different from samples not transfected with receptor; *, significantly different from samples transfected with the same receptor but not incubated with T_3.

To determine if transcriptional coregulators were involved in the ability of TRα and T_3/TRα to regulate cyp7a promoter activity, a strategy of site-directed mutagenesis was employed. After a review of the literature, leucine-213 in rat TRα was chosen for substitution. Leucine-213 is part of a conserved, hydrophobic groove which is required for both coactivator and corepressor interactions[184]. In TRβ, alanine-substitution of the homologous leucine residue (L213A) prevents the interaction between the receptor and an interacting domain of the SMRT corepressor *in vitro*. In addition, leucine-213 is conserved in TRα and TRβ from various species as well as in PPARs and RXRs. Thus, a mutant

TRα expression vector encoding an alanine substitution of leucine 213 (TRα-L213A) was created. Unlike the wild type receptor, mutant TRα failed to increase cyp7a promoter activity in RH7777 cells (Figure 3-7) suggesting that coregulator-binding to ligand-free TRα-L213A is defective. TRα-L213A was still able to reduce cyp7a promoter activity in the presence of T_3, but this effect was reduced compared to the wild type TRα as indicated by the fold-repression (TRα, 1.3 vs. TRα-L213A, 0.6). Thus, coregulator interactions appear to be important for T_3-mediated repression of promoter activity.

3.2.7 The murine Cyp7a1 proximal gene promoter is not repressed by T_3 – The results above suggest that human *CYP7A1* gene expression is repressed by T_3. In contrast, i*n vivo* experiments in rats and mice show that cyp7a mRNA abundance and *Cyp7a1* gene expression are increased by T_3[83,85-88]. One explanation for this discrepancy is that TRα binds and transactivates the rodent promoter *via* a different TR-binding sequence. Previous reports have shown that a promoter-reporter gene chimera containing approximately 3600 nt of the rat *Cyp7a1* gene promoter failed to respond to T_3 in HepG2 cells cotransfected with an expression vector encoding TR[66]. However, this may an artifact due to testing the activity of a rodent promoter in a human cell line. Thus, a promoter-reporter gene chimera containing the proximal murine *Cyp7a1* gene was transfected into RH7777 cells (which are of rat origin). While the human cyp7a promoter-reporter gene chimera responded as expected (transrepression), the activity of the murine cyp7a promoter was not affected by T_3 (Figure 3-8A). To ensure that the murine

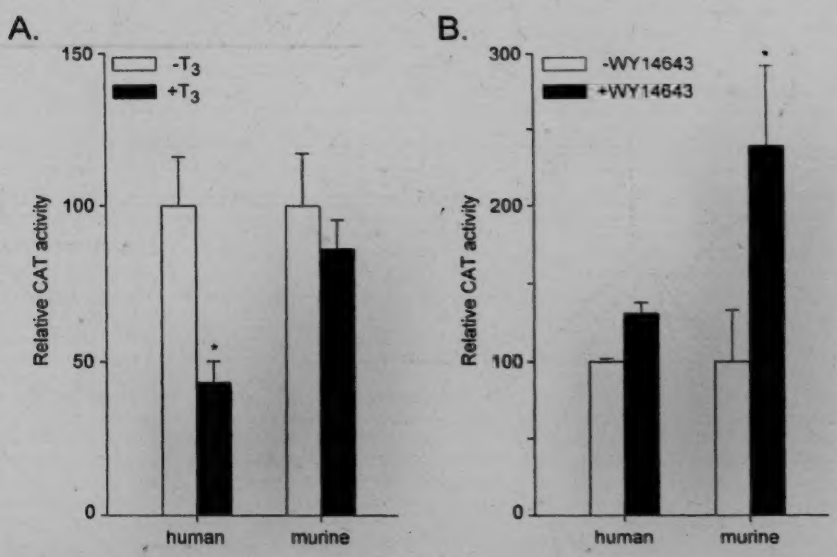


Figure 3-8. THE MURINE *CYP7A1* GENE PROMOTER IS NOT AFFECTED BY T_3. RH7777 cells were co-transfected with TRα (panel ***A***, complete media) or PPARα/RXRα (panel ***B***, complete media diluted 1:1 (v/v) with DMEM) and either the human or murine cyp7a promoter-reporter gene chimeras. Transfected cells were then grown for 18 hours in the presence of carrier (open bars), 100 nM T_3 (panel A, filled bars) or 50 μM WY14643 (panel B, filled bars) and assayed for CAT activity. The activities of the promoters in the absence of T_3 (panel A) or WY14643 (panel B) were taken as 100%. The data shown are the mean ± SEM relative CAT activity of triplicate assays from at least two experiments. Differences were evaluated using a two-sample *t*-test and were considered significant when $P<0.05$. *, significantly different from samples transfected with the same cyp7a gene chimera but not incubated with T_3 or WY14643.

cyp7a promoter was active in these cells, the ability of a PPAR-agonist, WY14643, to stimulate murine cyp7a in the presence of expression vectors encoding PPARα and RXRα was tested. Consistent with previous studies in our lab[55], WY14643 stimulated murine cyp7a promoter activity by 139% but showed only a modest ability to affect the human cyp7a promoter (Figure 3-8B). Thus, the murine cyp7a promoter can be regulated in RH7777 cells, but its activity is not affected by T_3/TRα.

The analysis of the human *CYP7A1* gene promoter presented herein identified a specific sequence in Site III which was required for both TRα-binding

as well as T_3-mediated repression of promoter activity. If the ability of T_3 to repress the promoter is directly related to TRα binding, the murine promoter should not bind TRα at Site III because T_3 does not affect murine *Cyp7a1* gene promoter activity. DNAse I footprinting of the murine cyp7a promoter in the presence of TRα did not reveal any receptor binding at Site III (Figure 3-9A). However, receptor binding at Site II was observed in the murine cyp7a promoter, similar to the human cyp7a promoter (Figure 3-9B). This result is expected as Site II is highly conserved between species. Additionally, TRα appears to bind the murine but not the human promoter at Site I (Figure 3-9C). However, this *in vitro* binding does not appear to be transcriptionally productive as the activity of the murine promoter was not affected by T_3 in RH7777 cells. No other binding sites were observed in the proximal murine *Cyp7a1* gene promoter.

3.3 *Discussion*

Thyroid hormones play important roles in development and energy homeostasis by regulating the transcription of a variety of target genes *via* TRs. The two major isoforms of the TRs (TRα and TRβ), which are encoded by two distinct genes, are expressed in a wide variety of tissues. Analysis of the expression of the TRα and TRβ genes revealed a disparity in the abundance of their mRNAs. However, quantitative indirect immunofluorescence studies have revealed that TRα and TRβ proteins are present in similar quantities in the nuclei of rat hepatocytes[185]. Additionally, an examination of TR abundance in human

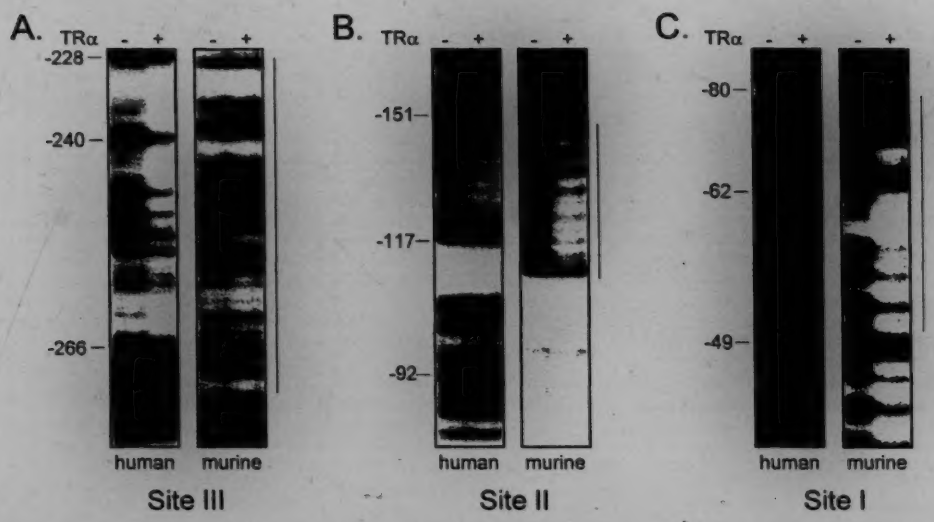


Figure 3-9. MAPPING TRα BINDING SITES IN THE MURINE *CYP7A1* GENE PROMOTER. Human *CYP7A1* or murine *Cyp7a1* gene promoter fragments were radiolabeled, incubated with or without recombinant TRα and partially degraded with DNAse I. The products were analyzed by denaturing electrophoresis on a 6% polyacrylamide gel and the corresponding autoradiograms are shown. The homologous regions of Site III (panel ***A***), Site II (panel ***B***) and Site I (panel ***C***) in the human and murine promoters are displayed. The corresponding positions in the human *CYP7A1* gene promoter are indicated at left.

liver indicated that TRα and TRβ are present in comparable amounts[186], suggesting that both isoforms are readily available for T_3-dependent gene regulation. Further experiments are required to determine if TRα and TRβ are differentially utilized *in vivo* to regulate human *CYP7A1* gene expression.

Sites II and III in the human *CYP7A1* gene promoter were identified as targets of TRα-binding by DNAse I footprinting, and EMSAs confirmed that two molecules of TRα can bind each site. The invariant nature of the Site II sequence among the five species examined to date suggested that this site would be important in the transcriptional regulation of the human *CYP7A1* gene promoter by T_3/TRα. Surprisingly, functional analysis revealed that T_3/TRα-dependent down-regulation of the human *CYP7A1* gene promoter does not involve this site.

Site II has been previously characterized as a focal point for the binding of several transcription factors to the human *CYP7A1*, mouse and rat *Cyp7a1* gene promoters[54,55,187-190]. This site may be occupied by other transcription factors *in vivo* and in cultured cells and therefore may not be available for binding of and regulation by T_3/TRα.

Site III is a novel regulatory region in the human *CYP7A1* gene promoter. It consists of multiple, overlapping hexanucleotide sequences which are putative binding sites for members of the nuclear receptor superfamily. Unlike Site II, the sequence of Site III is not conserved among different species, and the lack of binding of TRα to Site III in the murine promoter may partly explain the divergent responses of the human *CYP7A1* and rodent *Cyp7a1* genes to T_3. The stimulation of the rodent genes by T_3 may be due to indirect mechanisms or other TR-binding sites beyond the proximal promoter region.

TR may optionally utilize RXR when binding to regulatory sequences in target genes. When Site III was incubated with both TR and RXR, no heterodimeric complex was detected. Surprisingly, the addition of RXR resulted in the appearance of a more quickly migrating complex. Based on the subsequent analysis, it seems likely that this complex is a monomer of TR binding to Site III. Although the mechanism whereby RXR might induce the binding of TR monomers is not clear, it may be partly due to the higher concentration of RXR in the reaction mixture. The RXR expression vector produces approximately 5-fold more receptor than the TR expression vector. This is clearly illustrated *in vitro* as shown in Figure 2-3.

Indicators of bile acid synthesis from *in vivo* and *in vitro* experiments suggest that T_3 represses cyp7a activity in humans[90,128,130,156,157]. Consistent with this hypothesis, we found that the human *CYP7A1* gene promoter was inhibited by T_3. *In vitro* experiments suggest that repression of gene expression by TR homodimers may involve the recruitment of the SMRT corepressor[191]. Our data indicate that the molecular basis for the repression of the human *CYP7A1* gene promoter in response to T_3 involves the interaction of monomeric TRα with a novel element localized in Site III. Preliminary experiments suggest that T_3-dependent repression of human *CYP7A1* gene promoter activity requires the interaction of transcriptional coregulators with TRα at leucine 213. However, the expression and stability of mutant TRs in RH7777 cells should be analyzed to minimize artifacts which might result from an altered receptor structure.

This chapter describes the molecular basis for T_3/TRα-mediated repression of human cyp7a promoter activity. Ideally, these results would correlate with the response of the human *CYP7A1* gene to T_3 *in vivo*. However, these types of analyses are difficult in human subjects. To test this theory directly, a transgenic model of human *CYP7A1* gene expression in mice was created. This is the topic of Chapter 4.

Chapter 4

EXPRESSION OF THE HUMAN *CYP7A1* GENE IN TRANSGENIC MICE*

* Parts of this chapter have been published: Agellon LB, Drover VA, Cheema SK, Gbaguidi FG, and Walsh A (2002) *J Biol Chem* **277**:20131-20134.

4.1 INTRODUCTION

Our understanding of the regulatory mechanisms controlling cyp7a has been derived primarily from animal models. However, the regulation of cyp7a by T_3 appears to be different in humans and rodents (see Table 1-2). Unfortunately, cyp7a activity and *CYP7A1* gene expression are difficult to study *in vivo* in humans. To directly examine the effects of T_3 on human cyp7a, a transgenic mouse strain expressing the human *CYP7A1* gene was created. This chapter describes the construction, characterization and validation of this novel mouse strain.

4.2 RESULTS

4.2.1 A human genomic DNA clone contains the CYP7A1 *gene* – To study gene regulation in a transgenic model, it is imperative that the regulatory elements in the gene of interest be intact. Thus, a DNA fragment containing the entire human *CYP7A1* gene including the 5′- and 3′-flanking DNA sequences was used to create transgenic mice. The transgene DNA was obtained from a BAC library created from human genomic DNA which was commercially screened by PCR with primers specific to the 3′-untranslated region of the human *CYP7A1* gene. One clone, *CYP7A1*(BAC), was obtained from this screen and used for subsequent analysis.

To ensure that the human *CYP7A1*(BAC) contained both 5′- and 3′-flanking sequences, PCR analysis was performed using human-specific primers. As shown in Figure 4-1A, the human *CYP7A1*(BAC) produced 5′- and 3′-

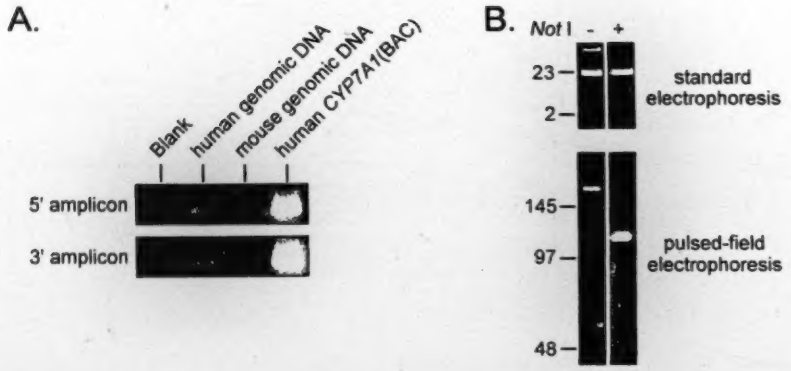


Figure 4-1. ISOLATION AND PURIFICATION OF THE HUMAN *CYP7A1* (BAC) CLONE. ***A,*** A clone containing the *CYP7A1* gene was obtained from a human genomic BAC library. The presence of the *CYP7A1* gene promoter (5' amplicon) and 3'-untranslated region (3' amplicon) were confirmed in this clone [*CYP7A1*(BAC)] by PCR using human-specific primers. Human and murine genomic DNA samples were also used as templates for PCR and represent the positive and negative controls, respectively. The lane labeled *Blank* represents reactions without template DNA. The amplicons produced were analyzed by agarose gel electrophoresis and detected by ethidium bromide staining. ***B,*** The human *CYP7A1*(BAC) clone was purified, digested with *Not* I and analyzed by gel electrophoresis following size exclusion chromatography through a Sepharose CL4B column. The DNA fragments were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. The size (kb) and position of DNA standards (standard electrophoresis, λ/*Hind* III; pulsed-field electrophoresis, λ concatamers) are indicated at the left.

amplicons similar in size to those produced from human genomic DNA templates. These primers are specific for the human *CYP7A1* gene as murine genomic DNA did not produce any amplicons.

At the inception of this project, the human *CYP7A1* gene had been cloned and was known to be about 12 kb[192]. To determine the size of the human DNA fragment in *CYP7A1*(BAC), restriction enzyme analysis was performed using the enzyme *Not* I. The recognition sequence of *Not* I is not common in mammalian DNA and this enzyme releases the insert from the BAC clone. Besides the BAC vector, only one DNA fragment was observed after *Not* I cleavage (Figure 4-1B, top panel) indicating that the genomic DNA insert did not contain any additional *Not* I sites. However, linearized insert displayed mobility similar to the 23 kb

marker and could not be resolved by standard gel electrophoresis. Analysis of the *Not* I-cleaved DNA by pulsed-field gel electrophoresis showed that the DNA fragment was approximately 120 kb (Figure 4-1B, bottom panel) and likely contained abundant flanking sequences in addition to the *CYP7A1* gene.

Figure 4-2A illustrates the location of the human *CYP7A1* locus on chromosome 8. The sequence of the *CYP7A1* locus was obtained from the public database and used to determine the exact arrangement of exons and restriction endonuclease sites. This is also illustrated in Figure 4-2A. Following restriction enzyme digestion and DNA blotting, bands of the expected size (see figure legend) were detected by membrane hybridization with the cyp7a cDNA (Figure 4-2B). Similar analyses using a human *CYP7A1* gene promoter fragment (5′ probe) or 3′-untranslated region (3′ probe) during hybridization revealed fragments of similar sizes but only those localized to the 5′- and 3′-regions of the *CYP7A1* gene, respectively. This allowed us to determine the relative positions of each restriction fragment in the *CYP7A1* gene and confirmed the presence of at least 17 kb of 5′- and 6 kb of 3′-flanking DNA sequences. Together, these data illustrate that the BAC clone contained the entire human *CYP7A1* gene and very likely the bulk of the regulatory sequences.

4.2.2 Mice carrying the CYP7A1*(BAC) transgene display the human cyp7a mRNA and enzyme* – After confirmation of the structure of the human *CYP7A1*(BAC) clone, transgenic mice were produced by injecting the entire BAC

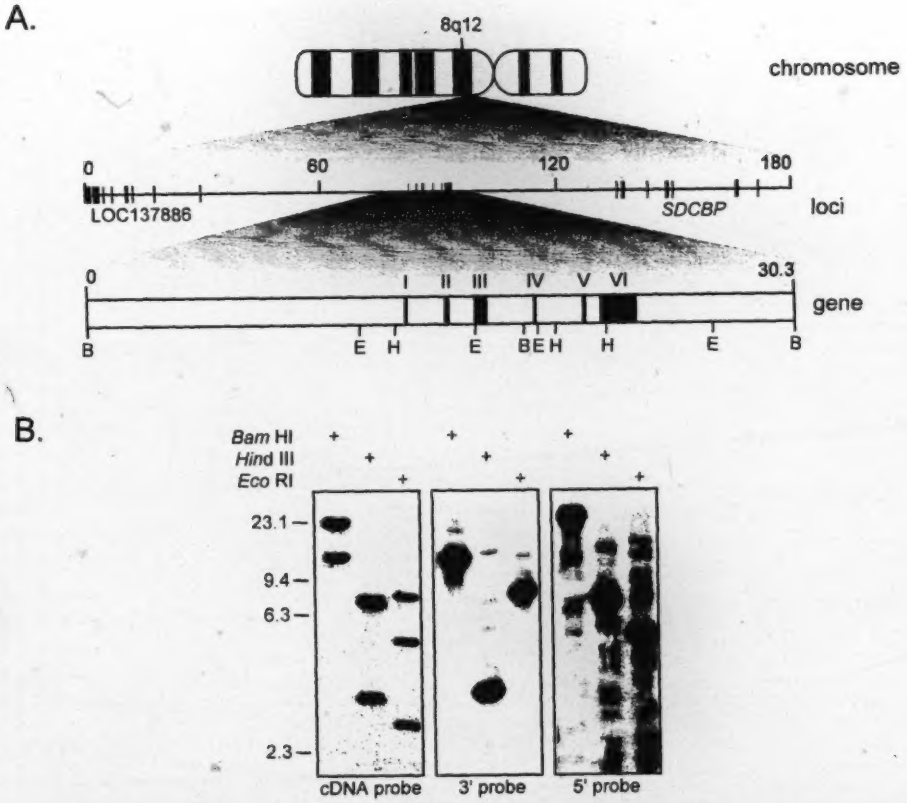


Figure 4-2. CHROMOSOMAL LOCATION AND RESTRICTION ENZYME ANALYSIS OF THE HUMAN *CYP7A1*(BAC). ***A,*** The typical banding pattern (G-bands) of human chromosome 8 is illustrated and the location of the *CYP7A1* gene is shown. Exons within the *CYP7A1* and adjacent loci are indicated by red boxes. The structure of the *CYP7A1* gene flanked by *Bam* HI restriction endonuclease sites is highlighted. Exons are labeled in Roman numerals above the gene and the position of restriction endonucleases sites (only those which produce DNA fragments containing exon sequences) are indicated in uppercase typeface below the gene (B, *Bam* HI; E, *Eco* RI; H, *Hind* III). The loci and *CYP7A1* gene is drawn to scale which is indicated in kb above the loci and the *CYP7A1* gene. ***B,*** Human *CYP7A1* (BAC) DNA was incubated with the restriction endonucleases shown and the DNA fragments separated by agarose gel electrophoresis. The autoradiograms produced after DNA blotting and solution hybridization with the indicated radiolabeled probes are shown. The expected sizes of DNA fragments containing *CYP7A1* exon sequence are: *Bam* HI, 18.8 kb, 11.6 kb; *Eco* RI, 7.5 kb, 5.0 kb, 2.7 kb; *Hind* III, 6.9 kb, 3.2 kb.

into C57BL6/J x CBA zygotes using standard procedures[xii]. Sixty potential founders (F_0) were produced and screened for the 5'-amplicon of *CYP7A1*(BAC) using the PCR method described in Figure 4-1A. Nine transgenic (Tg) F_0 were identified using this method. Each Tg F_0 was crossed with C57BL6/J and the F_1 generation analyzed for germline transmission of the human *CYP7A1* gene. Four of the F_0 did not transmit the transgene to the F_1 or did not produce and any F_1 progeny. Of the remaining five F_0, the human cyp7a mRNA and enzyme were unambiguously detected only in mice derived from one F_0 (Figure 4-3, panels A and B, respectively). A comparison of cyp7a activity in Tg and non-transgenic

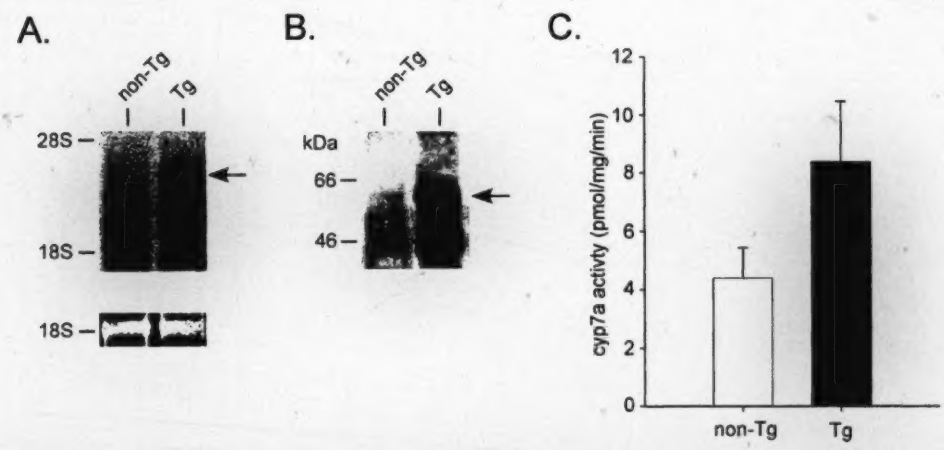


Figure 4-3. EXPRESSION OF THE HUMAN *CYP7A1*(BAC) IN MICE. Human *CYP7A1* gene expression was monitored in transgenic (Tg) mice created with the entire human *CYP7A1* (BAC) and non-transgenic (non-Tg) littermates. ***A,*** RNA blot analysis of hepatic cyp7a mRNA (arrow) using a DNA fragment corresponding to the 3'-untranslated region of the human *CYP7A1* gene. The positions of 28S and 18S ribosomal RNA are indicated on the left and the ethidium bromide-stained 18S ribosomal RNA is shown below. ***B,*** Cyp7a enzyme (arrow) was detected with antiserum against the last 5 carboxyl terminal amino acid residues of the human cyp7a enzyme. The positions of molecular weight markers are indicated on the left. ***C,*** Cyp7a enzyme activity was measured in hepatic microsomes. Differences between non-Tg (n=3) and Tg (n=3) were not statistically significant (P=0.08).

[xii] The microinjection procedure was performed by Dr. A. Walsh, in the Transgenic Services Laboratory at Rockefeller University, New York, NY, USA. Founder mice were shipped to the University of Alberta for screening and colony expansion.

(non-Tg) F_1 littermates from this line of mice revealed a trend for increased activity (Figure 4-3, panel C). Thus, a colony was expanded from these Tg mice.

4.2.3 The CYP7A1*(BAC) transgene rescues the cyp7a-null phenotype and responds to dietary stimuli* – One potential problem of using the Tg mice described above to study human cyp7a was the presence of the endogenous murine cyp7a. It was difficult to predict what effect human and murine cyp7a would have on the expression or activity of each other *in vivo*. Thus, a male transgenic mouse (third generation from the founder mouse) was crossed with a commercially available female cyp7a-null mouse. The breeding diagram shown in Figure 4-4 illustrates the crosses required to produce transgenic mice carrying

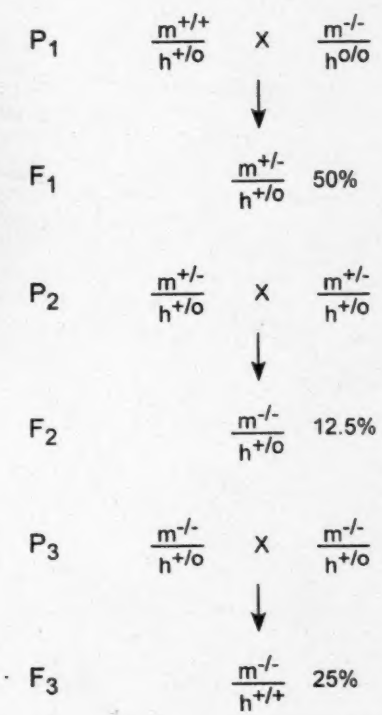


Figure 4-4. BREEDING DIAGRAM TO PRODUCE *CYP7A1* (BAC) TRANSGENIC MICE IN THE CYP7A-NULL BACKGROUND. Transgenic mice hemizygous for the *CYP7A1* (BAC) transgene and homozygous for the endogenous murine *Cyp7a1* gene ($m^{+/+}$ $h^{+/o}$) were crossed with cyp7a-null mice ($m^{-/-}$ $h^{o/o}$). The offspring were interbred until the desired, true-breeding genotype ($m^{-/-}$ $h^{+/+}$) was obtained. The genotypes of the parental (P) and the desired filial (F) generations are indicated. The hypothetical frequency of offspring containing the desired genotype in each cross is shown as a percentage.

the human *CYP7A1*(BAC) but lacking a functional allele of the endogenous *Cyp7a1* gene. The hypothetical percentage of the desired genotype after each cross is also shown.

Approximately 50% of the F_1 mice had the desired genotype (heterozygous for the murine *Cyp7a1* gene and hemizygous for the human *CYP7A1*(BAC)). This frequency dropped sharply (12.5%) when these F_1 mice were interbred to produce the desired F_2. However, mice homozygous for the murine cyp7a-null allele were very likely to contain the *CYP7A1*(BAC) as approximately 85% of cyp7a-null mice die in the first 18 days of life[133]. As shown in Figure 4-5A, the genotypes of the mice from the F_2 cross were determined by PCR (see the figure legend for the description of the primers used) and confirmed by DNA blotting and solution hybridization (Figure 4-5B), One male and one female mouse of the desired genotype were interbred to produce the F_3. The litter sizes of the F_3 were not noticeably different compared to wild type crosses suggesting that the expression of the human *CYP7A1*(BAC) in these *Cyp7a1*$^{-/-}$ mice was sufficient to prevent the perinatal mortality described above. In addition, the F_3 mice were overtly indistinguishable from wild type mice and did not display the skin or fur abnormalities observed in *Cyp7a1*$^{-/-}$ mice. Thus, a colony was expanded from the F_3 and used for further characterization. Hereafter, these mice will be referred to as Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$.

RT-PCR analysis of hepatic RNA from Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice revealed a specific product that was absent from *Cyp7a1*$^{-/-}$ mice (Figure 4-6A). The presence of a chimeric murine cyp7a/neo mRNA (which does not produce

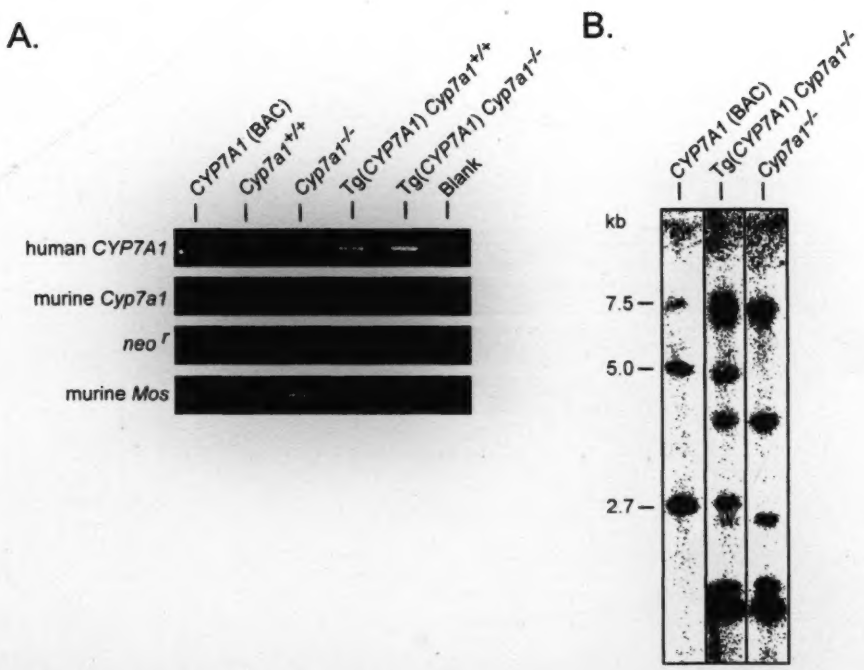


Figure 4-5. SCREENING OF TG(*CYP7A1*) MICE IN THE CYP7A-NULL BACKGROUND. ***A,*** The genotype of mice used in and produced from the breeding diagram shown in Figure 4-4 was determined by PCR. Oligonucleotides specific for the following genes were used: the human *CYP7A1* transgene, the wild type murine *Cyp7a1* gene, the neomycin phosphotransferase gene (neo^r) embedded in the disrupted *Cyp7a1* gene and the murine *Mos* gene (positive control target). Genomic DNA from the following mice was used as templates for the PCR reactions: wild type, *Cyp7a1*$^{+/+}$; murine cyp7a-null, *Cyp7a1*$^{-/-}$; human *CYP7A1* transgenic, Tg(*CYP7A1*)*Cyp7a1*$^{+/+}$; and murine cyp7a-null human *CYP7A1* transgenic, Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$. The lane labeled *Blank* represents reactions without template DNA. The amplicons produced by PCR were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. ***B,*** DNA blot hybridization patterns of the human *CYP7A1*(BAC), tail DNA of a mouse from the F_2 generation [Tg(*CYP7A1*)/Cyp7a1$^{-/-}$], and tail DNA of a mouse homozygous for the disrupted *Cyp7a1* allele (*Cyp7a1*$^{-/-}$). The blot was probed with a radiolabeled DNA fragment containing the rat cyp7a cDNA and the resultant autoradiogram is shown. The size (kb) of the *Eco* RI fragments of the human *CYP7A1* gene are indicated.

active cyp7a enzyme) was readily detected in both mice and is a well known feature of the cyp7a-null strain. Human cyp7a mRNA abundance was also determined in a number of tissues from a Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mouse. Figure 4-6B illustrates that the liver-specific expression of the human *CYP7A1* transgene is faithfully reproduced in mice. Previous reports have shown that increased cyp7a expression in the liver can reduce plasma cholesterol levels.

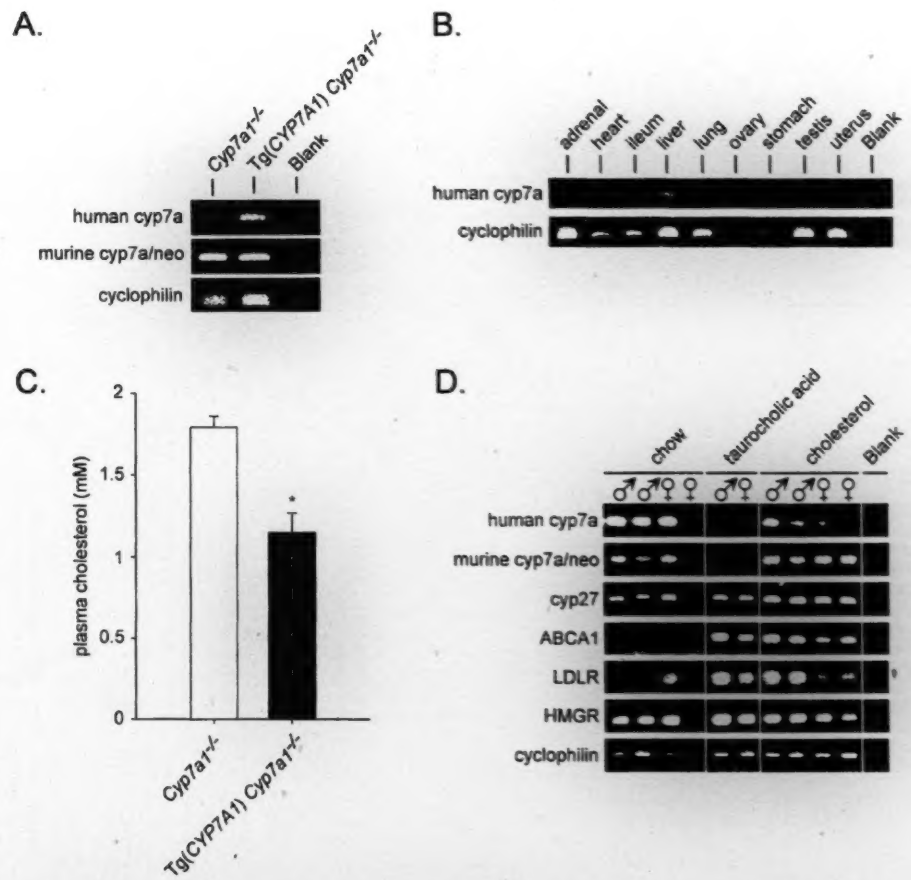


Figure 4-6. CHARACTERIZATION OF GENE EXPRESSION IN TG(*CYP7A1*)*CYP7A1*$^{-/-}$ MICE. ***A,*** Expression of the human *CYP7A1* gene and the murine *Cyp7a1-neo*^{r} gene chimera were confirmed by RT-PCR. See Figure 4-5 for a complete description of the genotypes shown. ***B,*** Expression of the human *CYP7A1* gene was detected by RT-PCR in a variety of tissues obtained from Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice. ***C,*** Male Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice and *Cyp7a1*$^{-/-}$ littermates (F_2) were deprived of food for 12 h and cholesterol levels were measured in plasma obtained from the tail vein. The data shown are the mean ± SEM and are representative of two experiments using mice from different litters (n≥3). * Differences were evaluated using the Student's *t*-test, *P*=0.005. ***D,*** Changes in the abundance of a number of hepatic mRNAs following dietary taurocholic acid (0.5% w/w) or cholesterol (1% w/w) were determined by RT-PCR in male (♂) and female (♀) Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice. The mRNAs analyzed were: human cyp7a, murine cyp7a/neo, cyp27, ATP binding cassette transporter protein A1 (ABCA1), LDL receptor (LDLR) and HMG CoA reductase (HMGR). In ***A***, ***B*** and ***D***, cyclophilin mRNA was used as a positive control in RT-PCR analyses and all lanes labeled ***Blank*** represent reactions without template DNA. The amplicons produced by PCR were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

Male Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice produced in the F_2 had significantly reduced plasma cholesterol levels in comparison to *Cyp7a1*$^{-/-}$ littermates (Figure 4-6C), again suggesting that the human cyp7a enzyme was active in the Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice.

To determine if the flanking DNA sequences in the *CYP7A1*(BAC) were sufficient to direct regulation of cyp7a, male and female Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice were fed a chow diet or a chow diet supplemented with taurocholic acid or cholesterol. As shown in Figure 4-6D, taurocholic acid was able to reduce the mRNA abundance of the human cyp7a and murine cyp7a/neo mRNAs. Cholesterol reduced the abundance of the human cyp7a mRNA and increased the murine cyp7a/neo mRNA. A number of other genes involved in cholesterol and bile acid metabolism was also examined in these animals. Notably, cholesterol increased cyp27 and ABCA1 mRNA abundance but not mRNA abundance of the LDLR nor HMGR.

Cholesterol-dependent repression of human cyp7a mRNA abundance has never been reported. Therefore, the above results were verified in a larger study. After reverse transcription, relative abundance of the human cyp7a mRNA was determined using quantitative real-time PCR. As shown in Figure 4-7A, human cyp7a mRNA abundance was significantly reduced by dietary cholesterol in Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice while the murine cyp7a/neo and cyp27 mRNAs were stimulated. No significant change was observed in the LDLR mRNA abundance. Additionally, hepatic cyp7a activity was significantly reduced in Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice fed a chow diet supplemented with cholesterol

(Figure 4-7B).

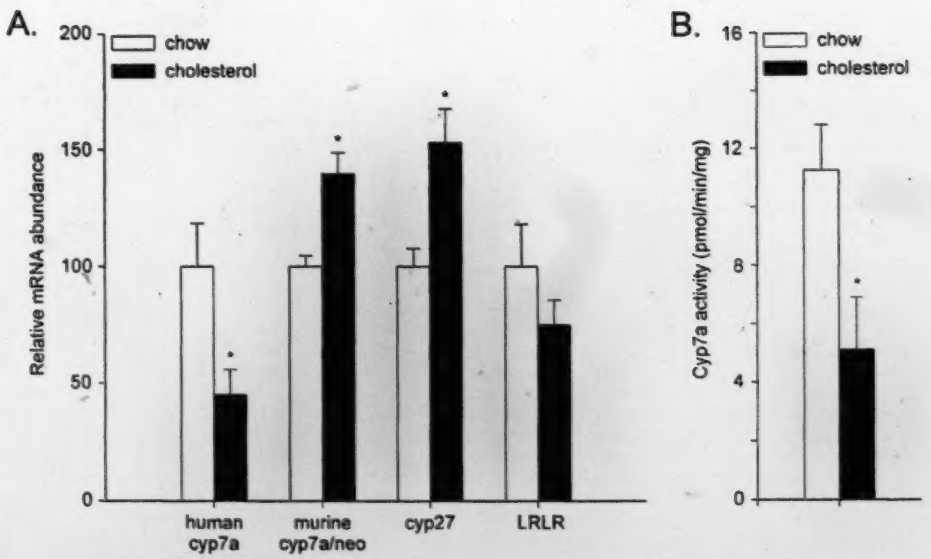


Figure 4-7. EFFECT OF CHOLESTEROL ON MRNA ABUNDANCE AND CYP7A ACTIVITY IN TG(*CYP7A1*)*CYP7A1*$^{-/-}$ MICE. ***A,*** Real-time PCR was used to quantitated the relative abundance of four hepatic mRNAs from Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice (male and female combined) fed a standard chow diet or a chow diet supplemented with 1% (w/w) cholesterol. The relative mRNA abundance in mice fed a chow diet was taken as 100%. See Figure 4-6 for a complete description of the mRNA symbols. ***B,*** Hepatic cyp7a activity was measured in Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice fed a standard chow diet or a chow diet supplemented with 1% (w/w) cholesterol. The data shown are the mean ± SEM of two experiments ($10 \leq n \leq 12$ total). * Differences were evaluated using the Student's *t*-test and considered significant when $P \leq 0.05$.

4.2.4 The human CYP7A1 *promoter is not stimulated by ligand-activated LXRα in vitro*[xiii] – In rodents, dietary cholesterol stimulates *Cyp7a1* gene expression presumably *via* increased oxysterol production. The oxysterols activate LXRs bound to Site I of the Cyp7a1 gene promoter resulting in transactivation of gene expression. The observation that cholesterol reduces

[xiii] These experiments were performed by a post-doctoral fellow in the laboratory, Dr. G.F. Gbaguidi.

human cyp7a mRNA and enzyme activity is not consistent with this mechanism. Thus, the response of the human cyp7a promoter-reporter gene chimera to ligand-activated LXRα was tested. When co-transfected into RH7777 cells with expression vectors encoding LXRα and RXRα, the activity of the human cyp7a promoter was not stimulated by 25-hydroxycholesterol, an LXR-agonist. In contrast, the activity of the murine cyp7a promoter-reporter gene chimera was significantly induced by 25-hydroxycholesterol. The inability of the human promoter to respond to liganded LXR was correlated to the inability of the human Site I sequence (see Figure 1-10) to interact with LXRα:RXRα in EMSAs (Figure 4-8B). The binding LXRα:RXRα heterodimers to the murine Site I sequence was readily detectable.

4.2.5 The sequence of Site I in the human CYP7A1 gene is conserved in a small cohort of volunteers – The published sequence of the human *CYP7A1* gene was used to design the oligonucleotides used in the EMSA analysis above. However, the *CYP7A1* gene promoter is known to contain polymorphisms[193,194]. To ensure that the sequence used in the EMSA analysis is representative of the population, genomic DNA samples were obtained from four unrelated individuals of various genetic backgrounds. The proximal *CYP7A1* gene promoters were amplified by PCR and sequenced directly. As shown in Figure 4-9, the sequence of Site I was invariant among these people. Sequence differences were detected at a known polymorphic site[193,194] elsewhere in the sequence which served as a positive control for the sequencing procedure. Thus, the published sequence of

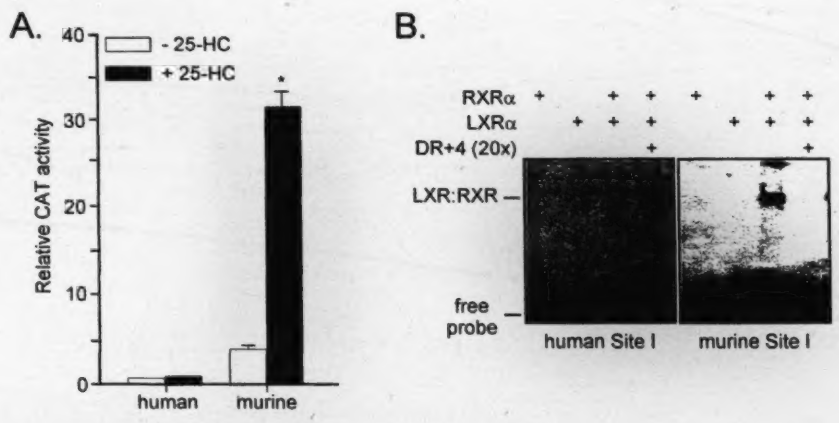
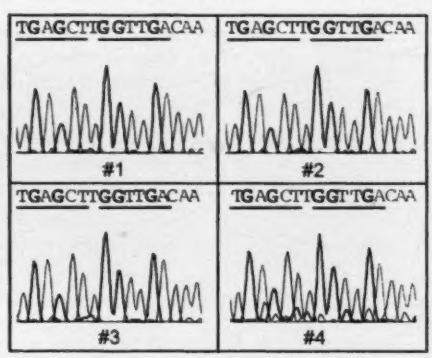


Figure 4-8. THE HUMAN *CYP7A1* GENE PROMOTER DOES NOT RESPOND TO LXR IN VITRO. ***A,*** Human *CYP7A1* and murine *Cyp7a1* gene promoter-reporter chimeras were transfected into RH7777 cells and treated with ethanol (carrier, open bars) or 5 μM 25-hydroxysterol (25-HC; filled bars). Following treatment, the cells were harvested and assayed for CAT activity. The data shown are the mean ± SD relative CAT activity of triplicate assays from two experiments. * Differences were evaluated using a one-tail *t*-test and were considered significant when $P \leq 0.05$. ***B,*** Labeled, double-stranded oligonucleotides corresponding to human or murine Site I of the cyp7a promoter were incubated with or without recombinant LXRα and RXRα. A double-stranded, idealized LXR-binding sequence (DR+4) was used as a competitor. Protein-DNA complexes were separated from free probe by non-denaturing electrophoresis on a 4% polyacrylamide gel. The migration of LXR:RXR heterodimers and the free probe are indicated at the left. These experiments were performed by a post-doctoral fellow in the laboratory, Dr. G.F. Gbaguidi.

Figure 4-9. THE HUMAN SITE I SEQUENCE IS INVARIANT IN FOUR INDIVIDUALS. Genomic DNA was prepared from peripheral leukocytes donated by four unrelated volunteers from different ethnic backgrounds. The *CYP7A1* gene promoter was amplified by PCR and the amplicons sequenced directly using an automated DNA sequencer in an on site facility. The sequence chromatogram spanning Site I is shown for each individual. The DR+1 is underlined.



human Site I in the *CYP7A1* gene promoter is likely representative of the population.

4.3 Discussion

The regulation of rodent and human cyp7a by T_3 may be different. While the effects of T_3 on rodent cyp7a mRNA abundance and enzyme activity are well characterized, the effects of T_3 on human cyp7a *in vivo* are largely unknown. To address this issue, a Tg mouse strain was created using a BAC clone containing a 120 kb human genomic DNA insert. This insert contained sequences flanking the exons of the human *CYP7A1* gene and restriction enzyme analysis was consistent with the known sequence of the human *CYP7A1* locus. Tg mice expressing human cyp7a were crossed with the *Cyp7a1*$^{-/-}$ mouse strain. The resultant Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice permitted the characterization of human *CYP7A1* gene expression without the functional consequences of the endogenous murine cyp7a. These mice also express a chimeric cyp7a/neo mRNA encoded by the disrupted *Cyp7a1*$^{-/-}$ allele. The abundance of cyp7a/neo mRNA was monitored and used as a control for the normal expression of the murine *Cyp7a1* gene.

Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice were overtly normal and did not require maternal diet supplementation with vitamins or bile acids. This observation illustrates that the level of *CYP7A1*(BAC) expression is high enough to support vitamin and fat uptake during perinatal development which is deficient in *Cyp7a1*$^{-/-}$ mice[133]. In addition, the expression of human cyp7a was regulated by

dietary stimuli in both male and female Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice. Taurocholic acid feeding reduced the human cyp7a and murine cyp7/neo mRNA below the detection limit of the RT-PCR procedure. This was expected as the sequence of the LRH-1 binding site is conserved among species and is present in the promoter of both the *CYP7A1*(BAC) and the disrupted murine *Cyp7a1*$^{-/-}$ gene.

Human cyp7a mRNA abundance was reduced by dietary cholesterol in Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice. This effect has never been reported in humans but is not entirely surprising as cyp7a mRNA abundance is reduced by cholesterol in primates[124]. The repression of the human *CYP7A1* gene was not likely due to murine strain differences in LXR/oxysterol signaling as the abundance of the murine cyp7a/neo mRNA was increased by cholesterol. The inability of cholesterol to induce human *CYP7A1* gene expression in Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice is likely due to the inability of LXR to bind and transactivate the promoter as shown above and by others[126]. Further, the Site I sequence of the *CYP7A1* gene in a small cohort of human subjects was invariant, suggesting that the lack of LXR-binding is a common feature in human populations. However, a lack of LXR-binding would only account for a lack of stimulation of cyp7a and not the additional reduction in mRNA abundance and activity observed in Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice. The mechanism of cholesterol-dependent repression of gene expression thus requires additional research.

Dietary stimuli had additional effects on other genes involved in bile acid and cholesterol metabolism. Messenger RNA abundance of cyp27, the first step in the acidic pathway of bile acid biosynthesis (see Section 1.4.1), was increased

by cholesterol while taurocholic acid had no effect. This is consistent with diet-studies in rats[20] but the effects of dietary stimuli on cyp27 mRNA abundance in mice has not been examined in detail.

ABCA1 mRNA abundance in the liver was also increased by dietary cholesterol. This would be consistent with the role of ABCA1 in cholesterol efflux as well as the ability of LXR and oxysterols to regulate ABCA1[195,196]. ABCA1 expression and activity in the liver are a matter of some controversy. However, increased cholesterol efflux *via* ABCA1 might account for the observation that the LDLR mRNA was unchanged by dietary cholesterol.

In summary, a transgenic model of human cyp7a was created in mice lacking a functional murine *Cyp7a1* allele. The *CYP7A1*(BAC) transgene contains sufficient DNA sequences flanking the *CYP7A1* gene to direct liver-specific expression of human cyp7a. The expression of *CYP7A1*(BAC) is sufficient to rescue the phenotype of perinatal mortality observed in *Cyp7a1*$^{-/-}$ mice. Further, the expression of the transgene responds as expected to the major dietary regulators of cyp7a and is thus a valid model in which to study human cyp7a regulation.

Chapter 5

THYROID HORMONE REPRESSES THE HUMAN *CYP7A1* GENE PROMOTER IN A GENDER-SPECIFIC FASHION IN TRANSGENIC MICE*

* This data is currently being prepared for submission to the *J Clin Invest.*

5.1 INTRODUCTION

As shown in Chapter 3, the activity of the human *CYP7A1* gene promoter is repressed by T_3 in hepatoma cells. In addition, some *in vivo* studies suggest that cyp7a activity and bile acid synthesis are reduced by T_3 (see Sections 1.4.3 and 1.5.3). However, T_3-status does not affect bile acid output in some studies[159,160]. Thus, the regulation of human cyp7a by T_3 remains poorly understood. To address this controversy, the ability of T_3 to affect cyp7a mRNA abundance and activity was tested in the "humanized" Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice described in Chapter 4.

5.2 RESULTS

5.2.1 Induction of hypo- and hyperthyroidism in mice – Oral administration of methimazole/perchlorate, in addition to a low-iodine diet, reduces plasma T_3 levels in humans and mice[88]. We used this treatment with or without hormone replacement (Figure 5-1A) to induce hyper- and hypothyroid states, respectively, in Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice[169]. Plasma T_3 was almost undetectable in male hypothyroid mice (0.02 ± 0.10 pg/mL; Figure 5-1B). Hyperthyroid males displayed T_3 levels of 1.65 ± 0.18 pg/mL, almost 2-fold higher than euthyroid controls. In hypothyroid females, T_3 levels were reduced to 0.21 ± 0.10 pg/mL while hyperthyroid females displayed 4-fold higher T_3 than the euthyroid controls (3.52 ± 0.59). Thus, the effects of methimazole/perchlorate and T_3 replacement were similar in both the male and female mice. Interestingly, T_3 levels in hyperthyroid females were 2-fold higher than treatment-matched males.

Euthyroid females had similar levels of plasma T_3 as treatment-matched males (0.86 ± 0.17 pg/mL and 0.84 ± 0.21 pg/mL, respectively).

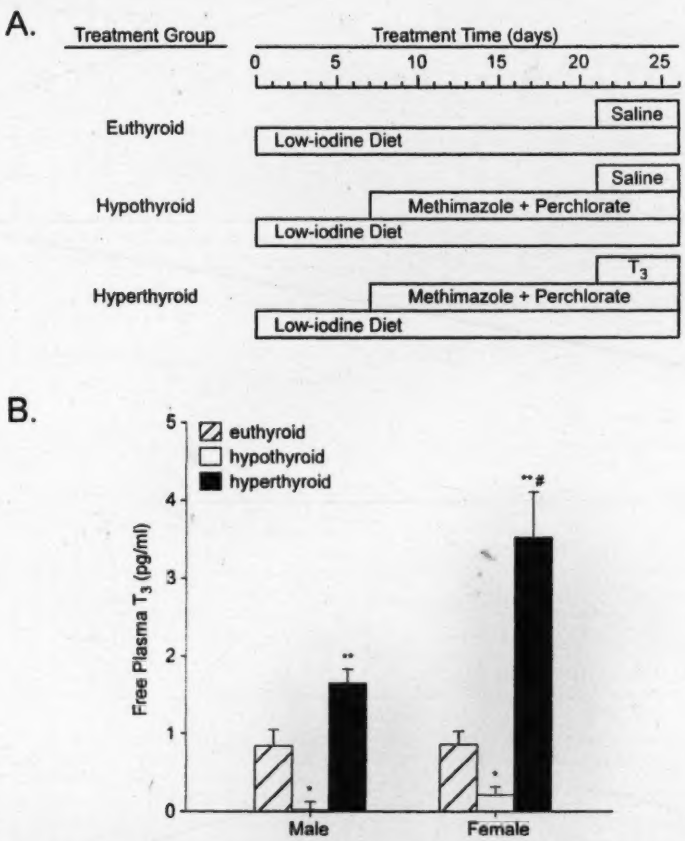


Figure 5-1. INDUCTION OF HYPO- AND HYPERTHYROIDISM IN MICE. ***A,*** The experimental manipulations to induce changes in plasma T_3 are illustrated. All mice were maintained on a low-iodine diet throughout the 26-day treatment period. Euthyroid controls were injected with saline only. The remaining mice received oral methimazole and perchlorate administration. The hypothyroid mice received saline injections while the hyperthyroid mice received injections of T_3. ***B,*** Free T_3 was measured in fresh plasma samples from transgenic mice ($7 \leq n \leq 10$). The data shown are the mean ± SEM from two experiments. Differences were evaluated using a two-sample t-test and were considered significant when $P<0.05$: * significantly different compared to euthyroid group, ** significantly different compared to both euthyroid and hypothyroid groups, # significantly different compared to treatment-matched males.

5.2.2 T_3 reduces plasma cholesterol levels and repartitions cholesterol into the HDL fraction in transgenic mice – In many mammals, hypothyroidism is associated with elevated total plasma cholesterol (TPC), a condition that can be reversed by hormone replacement[143]. As shown in Figure 5-2, both male and female hypothyroid mice had increased TPC compared to euthyroid mice. T_3 administration reduced TPC significantly in both sexes. There was no significant difference between euthyroid and hyperthyroid females.

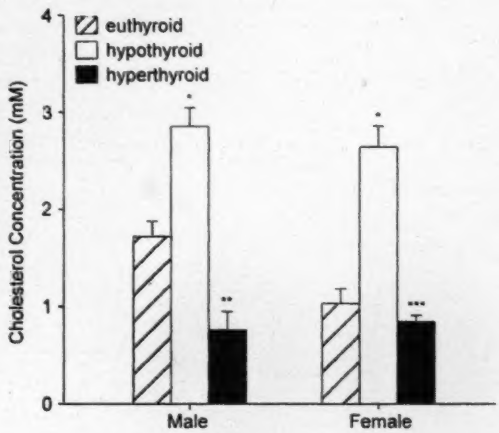


Figure 5-2. PLASMA CHOLESTEROL ANALYSIS IN HYPO- AND HYPERTHYROID MICE. Total cholesterol was measured in fresh plasma from transgenic mice ($7 \leq n \leq 10$). The data shown are the mean ± SEM from two experiments. Differences were evaluated using a two-sample t-test and were considered significant when $P<0.05$: * significantly different compared to euthyroid group, ** significantly different compared to both euthyroid and hypothyroid groups, *** significantly different compared to hypothyroid group.

The distribution of lipoprotein cholesterol is also affected by T_3 status. Hypothyroidism is typically associated with increased LDL-associated and HDL-associated cholesterol (LDL-C and HDL-C, respectively) mass, as well as an increase in the LDL-C:HDL-C ratio[197]. As shown in Figure 5-3A, hypothyroidism resulted in a dramatic increase in LDL-C mass which was reversed by T_3. HDL-C mass was also increased in hypothyroid mice and reduced in hyperthyroid mice. However, analysis of the distribution of cholesterol in each lipoprotein fraction expressed as a percentage of total lipoprotein-associated cholesterol revealed

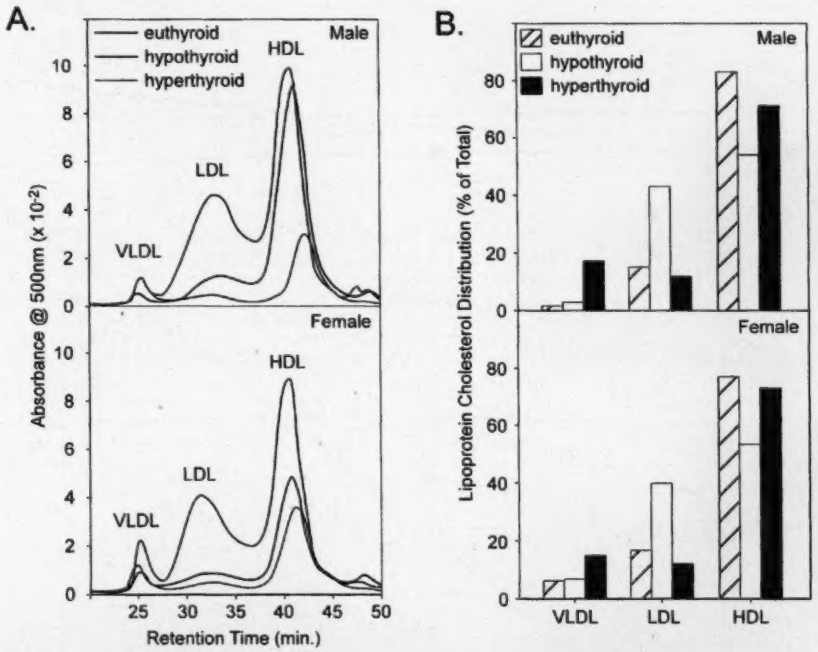


Figure 5-3. PLASMA CHOLESTEROL DISTRIBUTION IN HYPO- AND HYPERTHYROID MICE. ***A,*** Plasma samples were analyzed for cholesterol content following separation by gel filtration chromatography. The data shown were produced by pooling equal amounts of plasma from sex- and treatment-matched animals prior to analysis. ***B,*** The distribution of lipoprotein cholesterol was determined by quantitating the areas of the lipoprotein peaks shown in panel *A*. The amount of cholesterol in each lipoprotein class is expressed as a percentage of the total cholesterol.

that the percentage of HDL-C was reduced in hypothyroid mice while T_3 replacement reversed this effect (Figure 5-3B). These data illustrate that T_3 reduces both LDL-C and HDL-C mass. The remaining plasma cholesterol is preferentially partitioned to the HDL fraction.

T_3 had other noticeable effects on plasma lipoproteins. The percentage of very low density lipoprotein (VLDL)-associated cholesterol (VLDL-C) mass in hyperthyroid mice was increased more than 9-fold (males) and 2-fold (females) compared to euthyroid controls. Also, the HDL fraction in hyperthyroid mice had an increased retention time compared to the euthyroid and hypothyroid animals.

This was particularly evident in the male mice (hypothyroid, 40.8 min. vs. hyperthyroid, 42.5 min.).

5.2.3 Human cyp7a mRNA abundance is reduced by T_3 in male but not in female mice – We previously showed that the activity of the human *CYP7A1* gene promoter is repressed by T_3 *in vitro*[198]. To determine if the same effect occurs *in vivo*, we examined the relative abundance of human cyp7a mRNA in transgenic mice after induction of hypo- and hyperthyroid states. As shown in Figure 5-4A, human cyp7a mRNA levels in male mice were similar in the euthyroid and hypothyroid groups. Cyp7a mRNA abundance was reduced by approximately 40% in hyperthyroid mice, consistent with our *in vitro* results[198]. In contrast, human cyp7a mRNA abundance did not differ significantly in any of the female treatment groups. To determine if this gender disparity is specific for

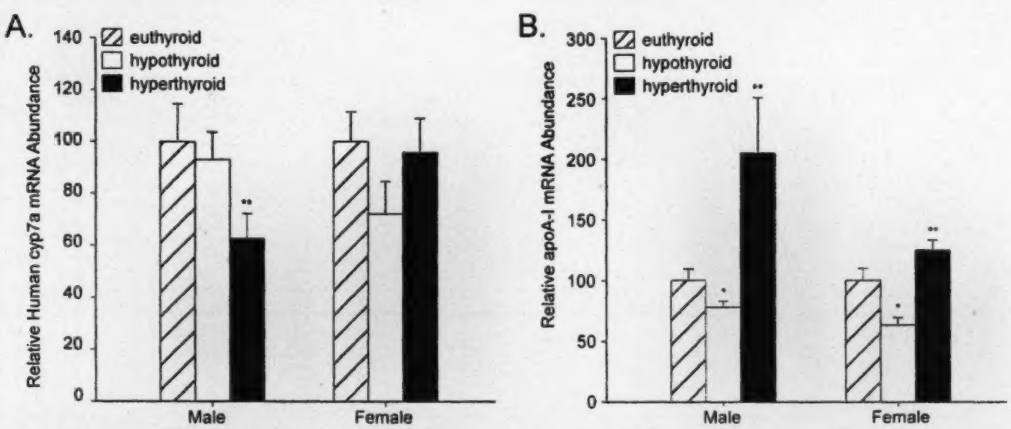


Figure 5-4. THE EFFECT OF T_3 ON HUMAN CYP7A AND MURINE APOA-I MRNA ABUNDANCE. The relative abundance of human cyp7a (panel ***A***) and murine apoA-I (panel ***B***) mRNAs were determined in transgenic mice ($7 \leq n \leq 10$). The data shown are the mean ± SEM from two experiments. Differences were evaluated using a two-sample t-test and were considered significant when $P<0.05$: * significantly different compared to euthyroid group, ** significantly different compared to both euthyroid and hypothyroid groups.

cyp7a, we monitored the changes in apoA-I mRNA abundance. As shown in Figure 5-4B, male hypothyroid mice had significantly reduced apo-AI mRNA abundance while hyperthyroid animals displayed mRNA levels 105% greater than euthyroid controls. Hyperthyroid females displayed a smaller (25%) but significant increase in apoA-I mRNA abundance. In male and female mice, the effects of hypothyroidism were comparable. Thus, the gender-related differences in the regulation of mRNA levels by T_3 are specific to cyp7a.

5.2.4 T_3 increases cyp7a enzyme activity in male but not female mice – To determine if enzyme activity and mRNA abundance covaried, we measured human cyp7a activity in the hypo- and hyperthyroid states. Euthyroid and hypothyroid Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice of both sexes had comparable activities regardless of gender (Figure 5-5A). However, cyp7a enzyme activity in

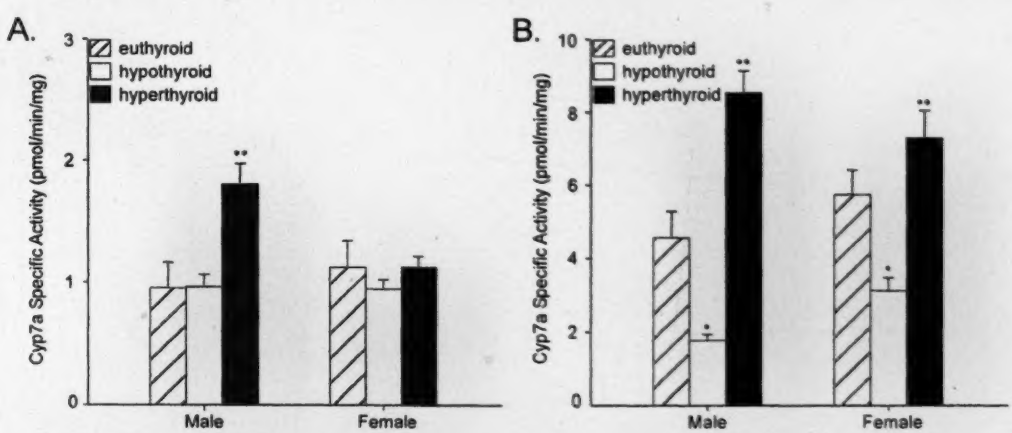


Figure 5-5. CYP7A ENZYME ACTIVITY IN HYPO- AND HYPERTHYROID MICE. Cyp7a specific activity was measured in hepatic microsomes prepared from transgenic (panel ***A***, $7 \leq n \leq 10$) or wild type (panel ***B***, n=4) mice. The data shown are the mean ± SEM from one (panel *B*) or two (panel *A*) experiments. Differences were evaluated using a two-sample t-test and were considered significant when $P<0.05$: * significantly different compared to euthyroid group, ** significantly different compared to both euthyroid and hypothyroid groups.

hyperthyroid animals was significantly increased 2-fold in males. No significant change was observed in female mice. Thus, cyp7a activity and mRNA abundance are negatively correlated in male mice but Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ positively correlated in female Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice. Cyp7a activity in wild type mice showed the expected T_3-dependent increase in activity in both male and female mice (Figure 5-5B). Thus, the gender differences observed in cyp7a activity are specific to Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice.

5.2.5 T_3-dependent changes of bile composition are gender specific – Bile acid pool size is inversely correlated with cyp7a activity[113,114]. In an effort to explain the sexual dimorphism of human cyp7a mRNA abundance and enzyme activity, we measured gallbladder bile acids in Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice as an indicator of bile acid pool size. Male hyperthyroid mice showed significantly reduced total gallbladder bile acid mass in comparison to male hypothyroid mice (Figure 5-6A). The concentration of bile acids was also reduced in male hyperthyroid mice (euthyroid, 53.46 ± 9.48 mM vs. hyperthyroid, 32.85 ± 5.21 mM, p=0.037). Thus, changes in total gallbladder bile acid mass were inversely correlated with changes in cyp7a activity in male mice. No significant change in bile acid mass or concentration in the gallbladder was observed in any of the female treatment groups.

Additional gender-specific effects were observed in the bile. Total gallbladder cholesterol mass was reduced in male hyperthyroid mice but not in females (Fig. 5-6B). However, female mice have more than twice the amount of

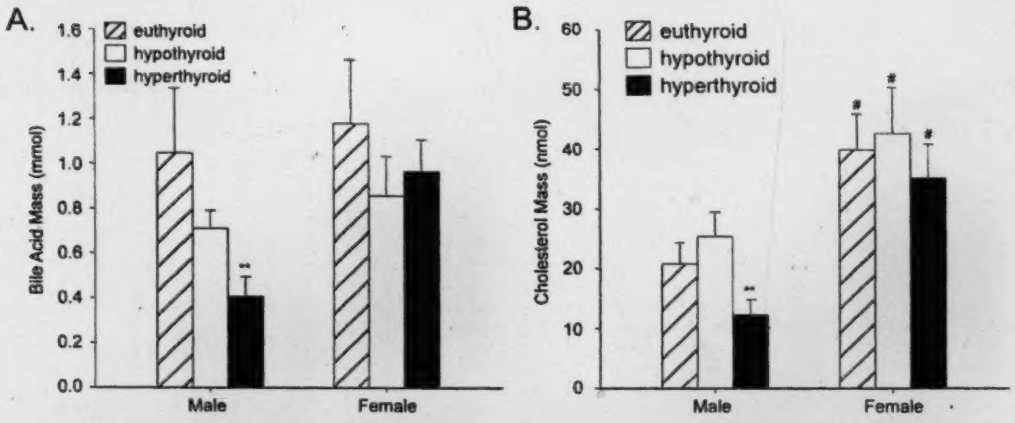
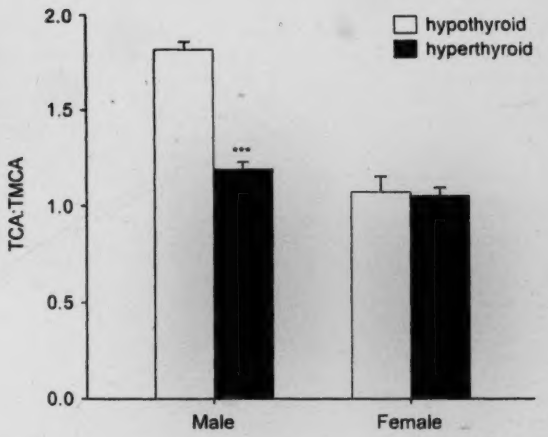


Figure 5-6. THE EFFECT OF T_3 ON BILIARY LIPIDS. Bile acid (panel ***A***) and cholesterol (panel ***B***) mass were measured in gallbladder bile from transgenic mice ($6 \leq n \leq 9$). The data shown are the mean ± SEM from two experiments. Differences were evaluated using a two-sample t-test and were considered significant when $P<0.05$: ** significantly different compared to both euthyroid and hypothyroid groups, # significantly different compared to treatment-matched males.

cholesterol in bile than male mice regardless of T_3-status resulting from increased in cholesterol concentration (euthyroid male, 1.22 ± 0.21 mM vs. euthyroid female, 2.83 ± 0.53 mM, p=0.009). Together, these data indicate that gender has a significant impact on bile acid metabolism in mice.

5.2.6 Gender differences in bile acid speciation are independent of cyp7a activity – To determine if the gender differences in human *CYP7A1* gene expression and bile composition were related to the transgene or other inherent factors in mice, we examined the ratio of gallbladder bile acids in wild type mice. As shown in Figure 5-7A, the ratio of TCA:TMCA was reduced by T_3 in wild type male mice but not in female mice. This gender disparity in wild type mice was not due to differences in cyp7a activity as T_3 increased enzyme activity to a comparable level in each gender (Figure 5-5B).

Figure 5-7. T_3 ALTERS BILIARY BILE ACID COMPOSITION AND CYP7A ACTIVITY IN MALE WILD TYPE MICE. The TCA:TMCA ratio in gallbladder bile was determined in wild type mice (n=4). The data shown are the mean ± SEM. Differences were evaluated using a two-sample t-test and were considered significant when $P<0.05$: *** significantly different compared to hypothyroid group.



In Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ male mice, the TCA:TMCA ratio was not significantly altered by T_3-status (Figure 5-8A). In contrast, hypothyroid females displayed a significantly increased TCA:TMCA ratio which was reversed with T_3 replacement. As cyp7a activity is increased in hyperthyroid males but unchanged in hyperthyroid females, the TCA:TMCA ratio cannot be accounted for by changes in cyp7a activity. Changes in sterol 12α-hydroxylase gene expression do not account for this difference in TCA:TMCA ratio. As shown in Figure 5-8B, T_3 caused a significant reduction in cyp8b mRNA abundance in male mice but a significant increase in females. Together, these data suggest that both wild type and Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice display inherent gender differences in bile acid speciation independent of cyp7a activity.

5.3 Discussion

The effects of thyroid dysfunction on bile acid synthesis in humans are poorly understood. In particular, the invasive techniques required for the

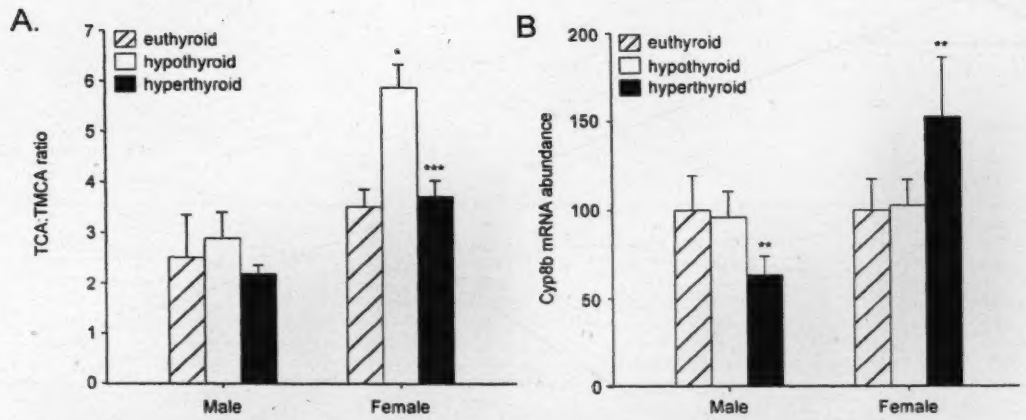


Figure 5-8. T_3 ALTERS BILE ACID COMPOSITION AND CYP8B MRNA ABUNDANCE IN A GENDER-SPECIFC FASHION IN TRANSGENIC MICE. The TCA:TMCA ratio in gallbladder bile (panel ***A***, 6≤n≤9) and the relative abundance of cyp8b mRNA (panel ***B***, 7≤n≤10) was determined in transgenic mice. The data shown are the mean ± SEM from two experiments. Differences were evaluated using a two-sample t-test and were considered significant when $P<0.05$: * significantly different compared to euthyroid group, ** significantly different compared to both euthyroid and hypothyroid groups, *** significantly different compared to hypothyroid group.

measurement of hepatic cyp7a enzyme activity and gene expression make direct analyses of human cyp7a difficult. To overcome this obstacle, we employed a transgenic mouse strain which expresses the human *CYP7A1* gene under the control of its natural regulatory regions but does not express its own endogenous *Cyp7a1* gene (Chapter 4). In this model the human cyp7a activity and *CYP7A1* gene expression can be tested without interference from the functional consequences of the endogenous enzyme. In the current report, we used hypo- and hyperthyroid states to study the ability of T_3 to regulate human cyp7a enzyme activity and mRNA abundance.

Pathological states which present with clinical T_3 deficiency are typically associated with hypercholesterolemia[199,200]. Plasma cholesterol concentration in rodents is also sensitive to T_3 status. Hypothyroid mice exhibited increased TPC

and repartitioned cholesterol into the LDL fraction. Thyroid hormone replacement reduced TPC levels and caused cholesterol to shift back to the HDL fraction. These data are consistent with the ability of T_3 to increase LDL catabolism via increased expression of the LDL receptor[153,201].

T_3-dependent changes in apoA-I mRNA abundance agreed with previous studies[155] and correlated with the percentage of HDL-C. However, it seems paradoxical to simultaneously increase apoA-I gene expression yet reduce HDL-C mass. One possible explanation is that cholesterol transport from the periphery to the liver *via* HDL has increased. Under these conditions, HDL-C uptake *via* scavenger receptor-B1 (the HDL receptor) should also be increased. However, the effects of T_3 on the activity of scavenger receptor-B1 have not been tested empirically.

Under conditions of increased apoA-I and reduced HDL-C, HDL particles should be smaller in size as they would contain less cholesterol. Apostolopoulos *et al.* showed that HDL particles in rats treated with T_3 were of smaller size but were enriched in apoA-I[155]. In addition, hypothyroidism in rats results in a larger HDL particle size[202]. Consistent with these reports, we observed smaller HDL particles in hyperthyroid mice as illustrated by the increased retention time of HDL in the lipoprotein cholesterol profiles. As apoA-I mRNA abundance is increased, it is possible that these smaller HDL particles are enriched in apoA-I compared to HDL in hypothyroid mice. Thyroid hormone can also increase hepatic lipase activity[203] which may further contribute to the reduction of HDL particle size by catabolizing the lipids in the lipoprotein.

Under euthyroid conditions, VLDL-C mass constitutes a minor proportion of the lipoprotein-associated cholesterol in mice. However, T_3 replacement increased VLDL-C mass to approximately 15% of TPC, comparable to LDL-C mass in this treatment group. Increased VLDL-C mass has also been observed in human hyperthyroid patients[197] while hypothyroid rats display reduced VLDL protein and VLDL-C mass[204]. Thus, increased VLDL-C is a characteristic feature of hyperthyroidism independent of species-specific differences in plasma cholesterol metabolism. The mechanism leading to increased plasma VLDL-C during hyperthyroidism is not known. However, increased cyp7a activity can increase the production of the active form of the sterol response element binding protein 1. This transcription factor is important in lipogenesis and possibly the provision of triacylglycerol substrate for VLDL secretion [reviewed in Kang and Davis (2000)[205]].

Human cyp7a mRNA abundance and the activity of the human *CYP7A1* gene promoter are reduced by T_3 *in vitro*[90,128,198]. Our finding that human cyp7a mRNA abundance is reduced by T_3 in male transgenic mice is consistent with these data and suggests that human *CYP7A1* gene expression can be repressed by T_3 *in vivo*. However, the role of gender in cyp7a regulation in transgenic mice was illustrated by the fact that T_3 had no effect on human *CYP7A1* gene expression in female mice. A number of gender differences in bile acid metabolism in rodents have been reported[206-209]. In contrast, gender differences in T_3 regulation of cholic acid synthesis or cyp7a activity has not been examined in a prospective study in humans. Retrospective analysis of the available clinical

data does not reveal a gender disparity in bile acid synthesis or output in male and female patients treated for hypo- or hyperthyroidism[130,156,157]. Thus, the inability of T_3 to affect cyp7a mRNA levels in female transgenic mice is likely due to gender differences in bile acid metabolism inherent in this rodent species. The observation that gallbladder cholesterol mass is two-fold greater in female mice than in male mice is consistent with this notion. Thus, secretion of free cholesterol may be a preferred mechanism for cholesterol removal from the female liver resulting in a reduced sensitivity of cyp7a to changes in hepatic cholesterol flux.

The effects of thyroid hormone on rodent cyp7a are mediated primarily at the level of transcription as cyp7a activity is tightly linked to mRNA levels and the rate of *Cyp7a1* gene transcription[85-88]. Human cyp7a enzyme activity was also increased by T_3 in male transgenic mice despite a reduction of cyp7a mRNA abundance. These observations suggest that post-transcriptional regulation of human cyp7a occurs *in vivo*. However, a T_3-induced increase of cyp7a activity is not consistent with the decreased rate of cholic acid synthesis/output observed clinically in some hyperthyroid patients[156,157]. One possible explanation is that the murine male liver, but not the female liver, requires increased bile acid synthesis in the hyperthyroid state and is capable of activating post-transcriptional mechanisms to stimulate human cyp7a activity when mRNA abundance is decreased. Reduced bile acid pool size and loss of feedback inhibition of cyp7a activity *via* a post-transcriptional mechanism is consistent with this hypothesis and the T_3-dependent changes in gallbladder bile acid mass reported here.

In summary, we report the novel finding that human cyp7a mRNA levels are reduced by thyroid hormone *in vivo*. In addition, post-transcriptional mechanisms and gender both contribute to the regulation of human cyp7a enzyme activity and biliary composition in mice.

Chapter 6

GENERAL DISCUSSION

SUMMARY

Bile acid biosynthesis is a critical metabolic function in mammals. It is the primary pathway for cholesterol catabolism and allows for the efficient uptake of dietary lipids from the gut. The neutral pathway of bile acid biosynthesis is regulated by cyp7a activity. In rodents, cyp7a activity is controlled primarily at the level of *Cyp7a1* gene expression and requires the interaction of promoter sequences with a host of transcription factors, many of which belong to the nuclear hormone receptor superfamily. The activity of these transcription factors may be regulated directly *via* ligand binding and receptor activation, or indirectly *via* interactions with additional regulatory proteins.

By utilizing indirect assays and indicators such as bile acid synthesis, a number of *in vivo* human studies report that cyp7a activity is reduced by T_3. The data herein illustrate that T_3 can also repress the activity of the human *CYP7A1* gene promoter *in vitro* in the presence of TRα. A novel regulatory sequence required for T_3/TRα-mediated repression was identified in the human *CYP7A1* gene promoter. In addition, T_3 reduced cyp7a mRNA abundance in transgenic mice expressing the human *CYP7A1* gene. Together, these data strongly suggest that *CYP7A1* gene expression can be repressed by T_3 in humans through a direct interaction of TR with the *CYP7A1* gene promoter.

PROMOTER-RECEPTOR INTERACTIONS AND TRANSCRIPTIONAL MECHANISMS

Putative recognition sequences of nuclear hormone receptors in the cyp7a promoter can be grouped by proximity into three regulatory regions: Site I, Site II

and Site III (Figure 1-10). In general, oxysterol and fatty acid regulation of cyp7a occurs at Site I. Liver-specific expression and feedback regulation by bile acids occurs at Site II. Diurnal expression and regulation by T_3 occurs at Site III. Species-specific variations in the sequence and utilization of these sites appear to be the basis of species-specific responses of cyp7a to different stimuli. For instance, Site II is highly conserved among species. Thus, permissive liver-specific expression and feedback inhibition by bile acids is a conserved feature of cyp7a regulation in mammals. In contrast, human and murine Site I display a number of sequence differences. The cyp7a1 gene promoters from these species also differ in their response to fatty acids/PPAR[55] and cholesterol/LXR (Chapter 4). Similarly, regulation of cyp7a promoter activity by T_3 is also species-specific (Section 3.2.7). The inability of T_3 to regulate the murine *Cyp7a1* gene promoter is likely due to sequences variations which prevent TR binding to Site III.

Additional TR binding sequences were identified in the human and murine cyp7a1 promoters. TR bound both the human and murine Site II sequence. Mutagenesis of this conserved sequence abrogated receptor binding *in vitro* but did not affect the ability of T_3 to repress the activity of the human *CYP7A1* promoter-reporter gene chimera. This indicates that human Site II is available for TR binding in EMSAs but not in cell culture, consistent with the permissive function of Site II in liver-specific gene expression. Because the sequence of Site II is conserved in humans and mice, it is likely that murine Site II is also occupied in RH7777 cells and not available for regulation by T_3/TR. TR also appeared to

bind murine Site I *in vitro*. It seems likely that TR cannot bind this sequence in RH7777 cells as the promoter does not respond to T_3/TR. *In vivo*, this site is probably occupied by LXR or PPAR.

The mechanisms required for transrepression of gene expression by T_3 are poorly understood. However, genes which are repressed by T_3-bound TR are generally induced by the unliganded aporeceptor [reviewed in Wu and Koenig, (2000)[210]]. Tagami *et al.*[211] demonstrated that nuclear corepressors were involved in transactivation of the genes required for T_3 synthesis (e.g. thyroid stimulating hormone) by unliganded TR. In addition, mice lacking the steroid receptor coactivator-1 displayed central resistance to T_3, resulting from an inability of T_3 to repress the expression of the thyroid stimulating hormone gene[212]. These data suggest that the roles of coactivators and corepressors may be reversed at genes which are negatively regulated by T_3. In addition, a model of transrepression by T_3/TR which is independent of DNA binding has been proposed for a subset of genes which are negatively regulated by T_3. In this model, the aporeceptor binds corepressors but not DNA. Thus, corepressors are sequestered away from the promoter and basal transcriptional machinery resulting in transactivation. T_3-bound TR sequesters coactivators and transrepression results as corepressors are liberated and made available to the promoter.

The molecular basis for T_3-dependent repression of the human *CYP7A1* gene promoter is the binding of a TR monomer to Site III. The mechanism requires TR binding to the DNA as mutations which disrupt receptor binding

interfere with the ability of T_3 to repress promoter activity. To determine if transcriptional coregulators were required, site-directed mutagenesis of TRα was employed. Figure 6-1 is a schematic representation of the structure of nuclear receptor ligand binding domains [reviewed in Aranda and Pascual (2001)[213]]. A conserved hydrophobic pocket created by helices 3, 4 and 5 is critical for coactivator and corepressor binding interactions with the receptor. Leucine 213 of TRα exists in this hydrophobic pocket between helices 3 and 4. The defective ligand-independent transactivation and T_3-dependent transrepression of TRα-L213A suggests that transcriptional coregulators are involved. Because leucine 213 appears to be required for the interaction of both types of coregulators with TR[184], the data does not discriminate between the roles of coactivators and corepressors in transrepression of the human *CYP7A1* gene promoter by T_3.

Recent studies by Gullberg *et al.* using TRα- and TRβ-deficient mice have

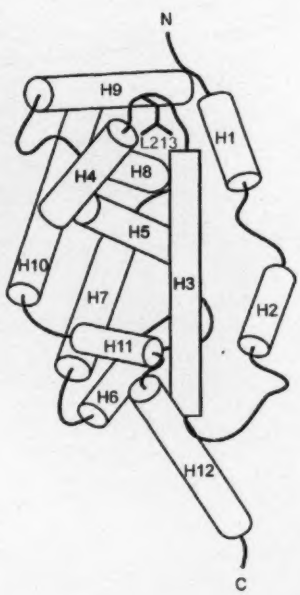


Figure 6-1. STRUCTURE OF A NUCLEAR RECEPTOR LIGAND BINDING DOMAIN. The crystal structure of the unliganded retinoid X receptor (ligand binding domain only) is shown schematically as a representative nuclear hormone receptor. α-Helices are indicated with cylinders and are numbered H1 through H12 relative to the amino terminus (N). The primary sequence of H3, H4 and H5 (indicated in filled cylinders) is highly conserved and forms a hydrophobic groove important for efficient interaction with coactivators and corepressors. The relative location of the conserved leucine (L213 in rat TRα) is indicated in red between helices H3 and H4. *Adapted from Aranda and Pascual (2001)[213].*

implicated TRβ in the T_3-dependent stimulation of *Cyp7a1* gene expression[88]. In this study, T_3 was able to induce cyp7a activity and mRNA abundance in TRα-null but not TRβ-null mice. However, TR did not bind murine Site III *in vitro*. Further, T_3 was unable to activate rodent *Cyp7a1* promoter-reporter gene chimeras in RH7777 or HepG2 cells[66]. There are a number of possibilities to explain this discrepancy. TR may bind upstream of the proximal promoter to regulate *Cyp7a1* in rodents. Because T_3 had no effect on the activity of a rat *Cyp7a1* promoter-reporter gene chimera consisting of the -3644 to +36 nt region of the gene, any positive TR binding sites would likely be upstream of -3644 nt.

Another explanation is that the ability of T_3 to induce rodent *Cyp7a1* gene expression is indirect. As discussed in Section 1.4.3(i), T_3 may potentiate the effects of glucocorticoids on *Cyp7a1* gene expression. This would be consistent with the inability of T_3 to regulate cyp7a mRNA abundance in isolated rat hepatocytes in the absence of glucocorticoids[89]. Alternatively, T_3 may increase the abundance of other nuclear receptors or ligands which induce *Cyp7a1* gene expression. Experiments with cholesterol-fed TRβ-null mice have shown that hepatic cholesterol is reduced by T_3[88]. As suggested by the authors, this may indicate that the production of oxysterol ligands for LXR is defective in TRβ-null mice. By extension then, T_3 may induce hepatic oxysterol production in wild type mice and thus induce *Cyp7a1* gene expression *via* LXR activation.

The treatment of Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice with dietary cholesterol has also uncovered a masked ability of cholesterol to repress *CYP7A1* gene expression. The data shown here do not allow a determination of the mechanism

of cholesterol-induced repression. However, recent work from our laboratory has shown that LXR can bind Site I of the murine *CYP7A1* gene promoter as a heterodimer with PPAR[214]. In addition, this LXR:PPAR heterodimer prevents transactivation of promoter activity by both LXR and PPAR ligands in RH7777 cells. Thus, increased LXR expression could interfere with PPAR-mediated stimulation of gene expression and *vice versa*. It is possible that a LXR:PPAR heterodimer binds to the human *CYP7A1* gene promoter. As LXR displays feed forward autoregulation by ligand-activated LXR[215], dietary cholesterol may repress human *CYP7A1* gene expression by increasing LXR expression. This in turn would result in increased LXR:PPAR heterodimer formation at Site I and thus reduced cyp7a mRNA abundance. More experiments are required to determine if this or other mechanisms contribute to cholesterol-mediated repression of human *CYP7A1* gene expression. In addition, the data suggest that the use of LXR-agonists to treat hypercholesterolemia may not have the desired effects in humans.

The use of Genetic models to study cyp7a

A range of genetic manipulations such as targeted gene disruption[13,133] has been applied to the study of cyp7a function and expression. Chimeric gene transfer[14,15,216] and transgenic expression[16,217] have also been used. However, these model animals do not permit the analysis of gene expression and enzyme activity in response to a stimulus as heterologous promoters are typically used to regulate transgene expression. The development of model systems to study the

regulation of gene expression has been hindered by the fact that all of the regulatory sequences required for detectable, liver-specific expression of the human *CYP7A1* gene have not been elucidated. This may have led to a number of failed attempts to create transgenic models expressing the human *CYP7A1* gene under the control of the natural promoter. This is illustrated by the observation that a transgene containing the sequence encoding the lacZ reporter fused to 1.8 kb of the human *CYP7A1* gene promoter was not expressed in the liver of transgenic mice[218]. Further, a transgene containing the human *CYP7A1* gene, 1.5 kb of promoter sequence and approximately 6.5 kb of DNA downstream of exon 6 displayed very low expression[218].

The Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice described herein were created using a BAC containing a 120 kb human genomic DNA fragment. The presence of approximately 17 kb of DNA upstream of the *CYP7A1* transcription start site and 6 kb of DNA downstream of exon 6 were verified by restriction enzyme analysis. The regulatory sequences in the *CYP7A1*(BAC) clone were sufficient to support liver-specific expression (similar to another report of human *CYP7A1* expression in transgenic mice by Goodart *et al.*[218]), as well as to rescue the perinatal mortality observed in cyp7a-null mice. The Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice also lack a functional murine *Cyp7a1* gene, thus facilitating the study of human cyp7a regulation without the influence of the endogenous enzyme. Although Goodart *et al.* state in their 1999 paper that they are

> "*in the process of generating mice that are homozygous knockouts for the endogenous Cyp7a1 gene and that contain one or more copies of the human gene*"[218],

experiments with these mice have not appeared in the literature. Thus, the Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice are currently a novel model of human *CYP7A1* gene expression.

An important step in the development of the Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice was the validation of gene expression using previously characterized stimuli. Liver-specific gene expression and feedback inhibition of mRNA levels by bile acids were observed suggesting that the transgene was functioning normally. Dietary cholesterol was also used to validate the model system. Previous experiments had shown that cyp7a activity and mRNA abundance in primates was reduced by cholesterol[124] and that LXR was unable to bind or activate the human *CYP7A1* promoter[126]. The data obtained from cholesterol-fed Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice was consistent with these reports and provided further evidence for the hypothesis that activated LXR induces murine *Cyp7a1* gene expression *via* binding at Site I.

The animal studies presented in Chapter 5 suggest that gender differences exist in human *CYP7A1* gene expression. Surprisingly, gender effects have essentially been ignored in the literature. For example, in 8 reports of the effects of T_3 on cyp7a in rodents, 7 used males[80,82,83,85-88] and only one used females[84]. None of the reports actually used animals of both genders. With respect to rodent cyp7a, T_3 does not display a gender bias (Takeuchi *et al.* (1975)[84] and Figure 5-5). However, gender differences in other aspects of bile acid metabolism were detected. Although cyp7a activity and gallbladder bile acid mass were comparable in male and female Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice,

gallbladder cholesterol mass was approximately 2-fold higher in females. The abundance of cyp8b mRNA also displayed gender differences in these mice. Further, the TCA:TMCA ratio displayed gender differences in both wild type and Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice. Together, these data provide a strong motivation for a careful analysis of the gender differences in bile acid metabolism in rodents and humans.

The Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice are currently the only non-human, *in vivo* model of human *CYP7A1* gene expression. Like most model systems however, it appears that the use of Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice to study human *CYP7A1* gene expression does have limitations. This was illustrated by the level of cyp7a activity. Cyp7a activity in chow-fed Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice was comparable to that observed in primates[124] and rabbits[122] but lower than chow-fed wild type mice. On a low iodine diet, the cyp7a activity in Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice was about 25% of that observed in wild type mice. Although cyp7a activity in Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice was sufficient to rescue the perinatal mortality observed in cyp7a-null mice, it is not clear how this activity compares to human liver due to the lack of available data from healthy human subjects. A direct comparison of bile acid pool size and contributions of the neutral and acidic pathways to this pool in wild type and Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice should also be performed to determine the effects of reduced cyp7a activity.

Another feature of the Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice that should be considered is the uncoupling of human cyp7a mRNA abundance and cyp7a activity in male hyperthyroid mice. While T_3 reduced human cyp7a mRNA

abundance, cyp7a activity was increased. This was somewhat unexpected as a large body of literature has shown that cyp7a mRNA and activity are usually correlated. However, two important implications can be drawn from this observation. Firstly, it appears that post-transcriptional mechanisms exist in the murine liver which can increase human cyp7a activity despite reduced mRNA levels. It has been known for some time that cyp7a activity can be regulated *in vitro* by phosphorylation [see Section 1.4.3(i)] and the activity of cyp7a may be regulated by T_3 in this way in male Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice. Secondly, the observation suggests that a T_3-dependent increase in cyp7a activity is necessary in murine liver. In other words, the normal signal for reduced cyp7a activity (i.e. reduced human cyp7a mRNA abundance) was ignored by the murine liver indicating a requirement for increased cyp7a activity. It is tempting to postulate that this metabolic requirement for enhanced bile acid synthesis may be a direct demonstration of an evolutionary pressure exerted by murine liver.

Do species-specific differences in hepatic cholesterol metabolism determine the effect of T_3 on CYP7A activity?

The ability of T_3 to repress human *CYP7A1* gene expression was confirmed *in vivo* in male Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice and is correlated with the ability of TR to bind the promoter at Site III. Sequence variation at this site appears to be responsible, at least in part, for the divergent responses of the human and rodent cyp7a1 genes to T_3. However, the purpose of reduced cyp7a activity and bile acid synthesis during hyperthyroidism in humans is unclear as T_3

has many similar effects in rodents and humans. For instance, T_3 increases *LDLR* gene expression and reduces plasma LDL-C levels in rodents and humans. In this scenario, it seems logical that bile acid synthesis should be increased to accommodate the increased hepatic uptake of LDL-C in both species. However, significant differences in hepatic cholesterol metabolism exist between humans and mice. Thus, sequence variation at Site III may have arisen from species-specific evolutionary pressures caused by differences in hepatic cholesterol metabolism.

A well characterized difference in hepatic cholesterol metabolism in humans and mice is the expression of apolipoprotein B (apoB), the main protein component of LDL and VLDL. Editing of the apoB mRNA leads to the translation of two proteins in murine liver: apoB100 is the full-length protein and contains 4563 amino acids while apoB48 is a truncated protein produced from the edited apoB transcript and contains 2152 amino acids [reviewed in Chan *et al.* (1997)[219]]. These large proteins require lipidation for efficient folding, stability and secretion from hepatocytes [reviewed in Shelness and Sellers (2001)[220]]. Using carboxyl terminal truncation mutants of apoB, Nicodeme *et al.* have shown that the lipidation required for apoB48 secretion is less than that required for apoB100[221]. As human liver lacks the apoB-mRNA editing complex and secretes apoB100 exclusively, apoB48 secretion from the murine liver should have a reduced requirement for lipid compared to apoB100 secretion from human liver. In addition, the expression apoB100, but not apoB48, in rat liver is repressed by T_3[222]. Could this species-specific difference result in distinct evolutionary

pressures on cyp7a? It is conceivable only if cholesterol is a limiting or regulatory component in the secretion of apoB-containing lipoproteins. Three lines of evidence support this notion. Firstly, over-expression of cyp7a in mice reduces apoB-containing lipoproteins in the plasma[16,216,217]. Secondly, apoA-I-induced cholesterol efflux in hepatocytes and RH7777 cells reduces the secretion of apoB48 and apoB100 as well the secretion of VLDL-associated triacylglycerol[223]. Thirdly, lovastatin treatment reduces cholesterol synthesis and VLDL secretion without affecting triacylglycerol synthesis or fatty acid oxidation[224,225]. These data suggest that cholesterol availability can be limiting in VLDL secretion. Thus, increased demand for cholesterol in human liver (due to the absence of apoB48 expression) may exert distinct evolutionary pressures on the human *CYP7A1* gene.

Enhanced VLDL secretion may be a common mechanism among mammals whereby triacylglycerol from the liver is delivered to the periphery as fuel. Consistent with this notion, VLDL-C can be increased up to 9-fold by T_3 in Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice. In the hyperthyroid state, VLDL-C mass is actually greater than LDL-C mass. A similar effect has been observed in other mammals, including humans. The observation that LDLR activity is increased during hyperthyroidism further suggests that VLDL secretion is enhanced (as opposed to reduced VLDL catabolism) when T_3 is in excess. If both humans and mice increase VLDL secretion in response to T_3, it is logical to assume that cholesterol will be needed in each species. As discussed above, cholesterol demand in human liver is likely to be less than in murine liver due to a lack of apoB48. In

addition, the availability of cholesterol for VLDL secretion may be lower in human liver. This concept may be deduced from known differences in hepatic cholesterol metabolism in humans and mice. The human liver synthesizes much less cholesterol than the murine liver (10 mg/kg/day vs. 160 mg/kg/day) and contributes about 4-fold less to whole animal cholesterol synthesis [reviewed in Dietschy and Turley (2002)[226]]. Further, a larger amount of cholesterol in humans is stored in the plasma as VLDL/LDL compared to mice (100 mg/dl vs. 7 mg/dl) due to the lack of cholesterol ester transfer protein in rodents.

Together, species-specific differences in the supply and demand of cholesterol for VLDL secretion provide a possible explanation for the species-specific differences in cyp7a regulation by T_3. When challenged with T_3, the murine liver increases cholesterol catabolism by stimulating cyp7a activity as excess cholesterol is available for VLDL secretion (Figure 6-2). This response helps to maintain the already high hepatic cholesterol levels in the face of increased LDL-C uptake *via* the LDLR. In contrast, cholesterol is less readily available and/or more cholesterol is required for VLDL secretion in human liver. When the human liver is challenged with T_3, cholesterol is conserved for VLDL secretion at the expense of bile acid synthesis and cyp7a activity is reduced. As increased LDL-C uptake is a common response to T_3 in both species and can lead to increased 27-hydroxycholesterol production in some tissues[227], it is possible that the 27-hydroxycholesterol concentration in human and murine hepatocytes is increased. Activation of LXR by 27-hydroxycholesterol may be the

mechanism whereby the expression of sterol regulatory element binding protein and VLDL secretion is increased[228] in both species.

To summarize, species-specific differences in hepatic cholesterol availability for VLDL secretion may be the source of the evolutionary pressures which lead to the divergent responses of human *CYP7A1* and murine *Cyp7a1* gene expression to T_3.

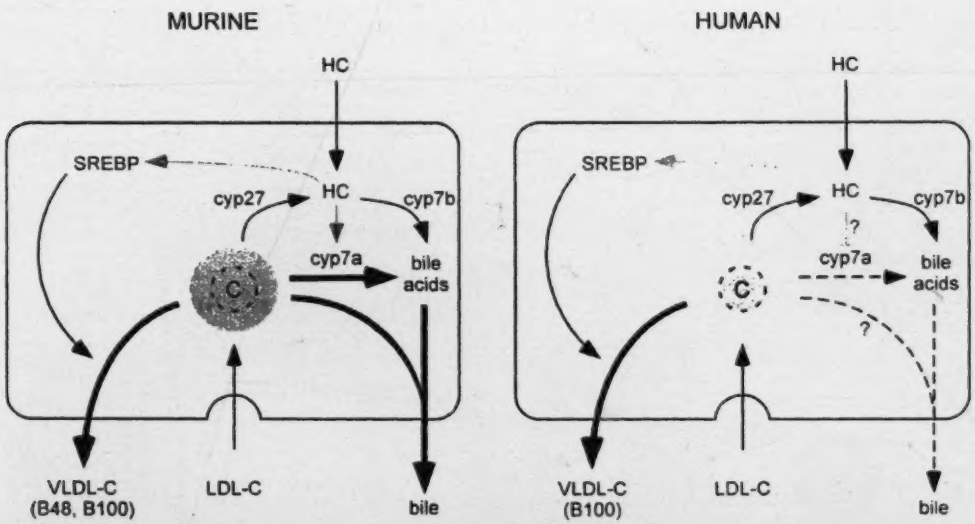


Figure 6-2. PUTATIVE MODEL OF STEROL METABOLISM IN HUMAN AND MURINE HEPATOCYTES DURING HYPERTHYROIDISM. The pool of cholesterol (C) available for VLDL secretion is indicated by the filled circle. In the murine hyperthyroid liver (*left*), this pool is in excess of the minimum amount of cholesterol required for VLDL secretion (dashed circle). Thus, VLDL-C secretion and cyp7a activity are both increased to prevent cholesterol accumulation. Increased concentrations of hydroxycholesterol (HC) resulting from enhanced LDL-C uptake or HC uptake from the plasma coordinate cyp7a activity and increased VLDL-C secretion. HC activates LXR which directly induces murine *Cyp7a1* (grey arrow) and indirectly increases VLDL-C secretion by inducing *SREBP* gene expression. In human liver (*right*), cholesterol availability is low during hyperthyroidism and cyp7a is reduced (dashed arrow) to preserve cholesterol for VLDL secretion. This is likely due to transrepression of T_3-bound TR at the *CYP7A1* gene promoter. HC levels are likely increased in human liver, thus inducing SREBP gene expression and VLDL-C secretion. HC may additionally repress human cyp7a *via* unknown mechanisms.

FUTURE DIRECTIONS

The work presented here raises a number of questions which require further investigation. The mechanism of T_3-dependent repression of *CYP7A1* gene promoter activity is a very interesting topic as mechanisms for negatively regulated genes remained elusive. Further, recent analysis of T_3-target genes using a hepatic cDNA array[229] found that the abundance of 41 mRNAs was reduced by T_3 while 14 mRNAs were increased. Thus, negative regulatory mechanisms for T_3 action may be more prevalent than originally thought. Mutagenesis of the DNA binding- and ligand-binding domains of TRα will verify that DNA and ligand binding are required for repression. *In vitro* binding assays using known transcriptional coactivators and corepressors may identify other proteins which are required for this effect. The mechanism whereby dietary cholesterol represses human *CYP7A1* gene expression also requires further investigation. As discussed above, LXRα may be required. This observation could easily be verified by crossing the Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice into the LXRα-null mouse and testing the response of the human *CYP7A1* gene to cholesterol. Another approach would be to create a transgenic mouse using a large murine genomic DNA transgene containing mutations at Site I which prevent LXRα binding. The response of this transgene to dietary cholesterol compared to the endogenous allele may also provide mechanistic clues.

An analysis of post-transcriptional regulation of human cyp7a in male hyperthyroid Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice is another exciting avenue of study. Post-transcriptional regulation by phosphorylation had a significant foothold in the

literature in the 1980s but very little has been published in the last 15 years since the correlation between cyp7a activity and mRNA abundance was realized. The Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice may be an excellent source of cyp7a enzyme with altered phosphorylation states as human cyp7a mRNA abundance and activity are uncoupled by T_3 in this strain. Given the large number of predicted phosphorylation sites, the best approach might be a large-scale purification of human cyp7a from hypo- and hyperthyroid mice Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice in the presence of phosphatase inhibitors. Protease digestion and mass determination of the resultant fragments might help to narrow down the location of any phosphorylated residues making a molecular biology approach more feasible.

The Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice should also prove useful in characterizing other differences in cyp7a regulation in humans and mice. For instance, the ability of fatty acids to regulate human cyp7a in the presence and absence of cholesterol can be readily tested in this model. The Tg(*CYP7A1*)*Cyp7a1*$^{-/-}$ mice may also be useful in the identification of drugs which may prevent hypercholesterolemia *via* increased cyp7a activity.

Extending the models proposed above may also provide insights into the physiological regulation of bile acid synthesis. For instance, if cyp7a is continually repressed during chronic hyperthyroidism in humans, the bile acid pool size will eventually be reduced below levels which support efficient lipid uptake from the intestine. In addition to T_3-mediated repression, the *CYP7A1* gene should experience multiple signals under these conditions such as loss of bile acid-

mediated repression and loss of ligand activated-PPAR regulation. Given the increased energy expenditure associated with hyperthyroidism, it is likely that cyp7a should be upregulated to maximize uptake of the energy-rich dietary lipids. Simultaneously administering T_3 and cholestyramine to Tg(*CYP7A1*)Cyp7a1$^{-/-}$ mice may allow for a direct testing of this hypothesis *in vivo*.

Concluding Remarks

Very little is known about the regulation of human cyp7a. The data presented in this thesis represent the first direct analysis of the ability of T_3 to regulate human cyp7a in an *in vivo* model and illustrates important differences in the regulation of rodent and human cyp7a. Specifically, the negative regulation of human cyp7a by T_3 and dietary cholesterol is opposite of that observed for murine cyp7a. These data further indicate that the human and murine liver have developed unique mechanisms to achieve the appropriate species-specific metabolic responses.

Chapter 7

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